(19) PATENT BUREAU OF JAPAN (JP)
(12) OFFICIAL GAZETTE FOR GRANTED PATENTS (A)

(11) Japanese Patent Application Publication Kokai: Sho 62-236497
(43) Publication Date: October 16, 1987
Number of Claims: 2
Request for Examination: Not requested (Total of 47 pages)

(51) Int. Cl.[4]	JP Class.	Intra-bureau Registration No.
C 12 P 21/02		6712-4B
A 61 K 37/02	ADV	8615-4C
C 07 K 13/00		8318-4H
C 12 N 15/00		7115-4B

(54) Title of Invention: Novel glycoprotein and method for producing it

(21) Patent Application No.: Patent Application No. Sho 61-166710
(22) Application Date: July 17, 1986

Priority Claims: (32) September 17, 1985 (33) Japan (JP) (31) Patent Application Sho 60-206066
(32) September 20, 1985 (33) Japan (JP) (31) Patent Application Sho 60-209638
(32) September 30, 1985 (33) Japan (JP) (31) Patent Application Sho 60-217150
(32) December 2, 1985 (33) Japan (JP) (31) Patent Application Sho 60-269456
(32) December 3, 1985 (33) Japan (JP) (31) Patent Application Sho 60-270839

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Detailed Description

1. Title of the Invention

Novel glycoprotein and method for producing it

2. Patent Claims

1. A glycoprotein having a polypeptide represented by the following amino acid sequence or a part of it and sugar chains, and having a human granulocyte colony-stimulating factor activity:

Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln
Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg
Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu
Lys Leu (Val Ser Glu)$_n$ Cys Ala Thr Tyr Lys
Leu Cys His Pro Glu Glu Leu Val Leu Leu Gly
His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser
Ser Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly
Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu
Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile
Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu
Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile
Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro
Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala

Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly
Val Leu Val Ala Ser His Leu Gln Ser Phe Leu
Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala

Gln Pro

(n is 0 or 1).

2. A method for producing the glycoprotein having a polypeptide represented by the following amino acid sequence or a part of it and sugar chains, and having a human granulocyte colony-stimulating factor activity, characterized by the fact that a gene encoding the polypeptide a having human granulocyte colony-stimulating factor activity is obtained, and then a recombinant vector is prepared containing the gene, followed by

transformation of the vector in a host cell, culture of the resultant transformant, and isolation of the glycoprotein from the culture:

Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln
Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg
Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu
Lys Leu (Val Ser Glu)$_n$ Cys Ala Thr Tyr Lys
Leu Cys His Pro Glu Glu Leu Val Leu Leu Gly
His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser
Ser Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly
Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu

Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile
Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu
Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile
Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro
Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala
Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly
Val Leu Val Ala Ser His Leu Gln Ser Phe Leu
Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala

Gln Pro

(n is 0 or 1).

3. The method for producing glycoprotein described under Patent Claim 2, characterized by the fact that the gene encoding the polypeptide having a human granulocyte colony-stimulating factor activity is a DNA complementary to a messenger RNA which is obtained by sucrose density gradient centrifugation as the 15-17 S fraction and encodes the polypeptide having a human granulocyte colony-stimulating factor activity.

4. The method for producing glycoprotein described under Patent Claim 2, characterized by the fact that the gene encoding the polypeptide having human granulocyte colony-stimulating factor activity is one derived from human chromosomes.

5. The method for producing glycoprotein described under Patent Claim 4, characterized by the fact that the human chromosome-derived gene contains nucleotide sequences involved in transcription regulation.

6. The method for producing glycoprotein described under Patent Claim 2, characterized by the fact that the gene encoding the polypeptide having a human granulocyte colony-stimulating factor activity encodes the following polypeptide sequence or a part of it:

Met Ala Gly Pro Ala Thr Gln Ser Pro Met
Lys Leu Met Ala Leu Gln Leu Leu Leu Trp His
Ser Ala Leu Trp Thr Val Gln Glu Ala Thr Pro
Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe
Leu Leu Lys Cys Leu Glu Gln Val Arg Lys Ile
Gln Gly Asp Gly Ala Ala Leu Gln Glu Lys Leu
(Val Ser Glu)$_n$ Cys Ala Thr Tyr Lys Leu Cys
His Pro Glu Glu Leu Val Leu Leu Gly His Ser
Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys
Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu
Ser Gln Leu His Ser Gly Leu Phe Leu Tyr Gln
Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser Pro

Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu
Asp Val Ala Asp Phe Ala Thr Thr Ile Trp Gln
Gln Met Glu Glu Leu Gly Met Ala Pro Ala Leu
Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala
Ser Ala Phe Gln Arg Arg Ala Gly Gly Val Leu
Val Ala Ser His Leu Gln Ser Phe Leu Glu Val
Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro

(n is 0 or 1).

7. The method for producing glycoprotein described under Patent Claim 2, characterized by the fact that the gene encoding the polypeptide having a human granulocyte colony-stimulating factor activity encodes the following polypeptide sequence or a part of it:

Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln
Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg
Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu
Lys Leu (Val Ser Glu)$_n$ Cys Ala Thr Tyr Lys
Leu Cys His Pro Glu Glu Leu Val Leu Leu Gly
His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser
Ser Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly

Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu
Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile
Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu
Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile
Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro
Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala
Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly
Val Leu Val Ala Ser His Leu Gln Ser Phe Leu
Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala

Gln Pro

(n is 0 or 1).

8. The method for producing glycoprotein described under Patent Claim 2, characterized by the fact that the gene encoding the polypeptide having a human granulocyte colony-stimulating factor activity encodes the following nucleotide sequence or a part of it:

ATG GCT GGA CCT GCC ACC CAG AGC CCC ATG
AAG CTG ATG GCC CTG CAG CTG CTG CTG TGG CAC
AGT GCA CTC TGG ACA GTG CAG GAA GCC ACC CCC
CTG GGC CCT GCC AGC TCC CTG CCC CAG AGC TTC
CTG CTC AAG TGC TTA GAG CAA GTG AGG AAG ATC
CAG GGC GAT GGC GCA GCG CTC CAG GAG AAG CTG

(GTG AGT GAG)$_n$ TGT GCC ACC TAC AAG CTG TGC
CAC CCC GAG GAG CTG GTG CTG CTC GGA CAC TCT
CTG GGC ATC CCC TGG GCT CCC CTG AGC AGC TGC
CCC AGC CAG GCC CTG CAG CTG GCA GGC TGC TTG
AGC CAA CTC CAT AGC GGC CTT TTC CTC TAC CAG
GGG CTC CTG CAG GCC CTG GAA GGG ATC TCC CCC
GAG TTG GGT CCC ACC TTG GAC ACA CTG CAG CTG
GAC GTC GCC GAC TTT GCC ACC ACC ATC TGG CAG
CAG ATG GAA GAA CTG GGA ATG GCC CCT GCC CTG
CAG CCC ACC CAG GGT GCC ATG CCG GCC TTC GCC
TCT GCT TTC CAG CGC CGG GCA GGA GGG GTC CTG
GTT GCC TCC CAT CTG CAG AGC TTC CTG GAG GTG
TCG TAC CGC GTT CTA CGC CAC CTT GCC CAG CCC

(n is 0 or 1).

9. The method for producing glycoprotein described under Patent Claim 2, characterized by the fact that the gene encoding the polypeptide having a human granulocyte colony-stimulating factor activity encodes the following nucleotide sequence or a part of it:

ACC CCC CTG GGC CCT GCC AGC TCC CTG CCC CAG
AGC TTC CTG CTC AAG TGC TTA GAG CAA GTG AGG

AAG ATC CAG GGC GAT GGC GCA GCG CTC CAG GAG

AAG CTG (GTG AGT GAG)$_n$ TGT GCC ACC TAC AAG

CTG TGC CAC CCC GAG GAG CTG GTG CTG CTC GGA

CAC TCT CTG GGC ATC CCC TGG GCT CCC CTG AGC

AGC TGC CCC AGC CAG GCC CTG CAG CTG GCA GGC

TGC TTG AGC CAA CTC CAT AGC GGC CTT TTC CTC

TAC CAG GGG CTC CTG CAG GCC CTG GAA GGG ATC

TCC CCC GAG TTG GGT CCC ACC TTG GAC ACA CTG

CAG CTG GAC GTC GCC GAC TTT GCC ACC ACC ATC

TGG CAG CAG ATG GAA GAA CTG GGA ATG GCC CCT

GCC CTG CAG CCC ACC CAG GGT GCC ATG CCG GCC

TTC GCC TCT GCT TTC CAG CGC CGG GCA GGA GGG

GTC CTG GTT GCC TCC CAT CTG CAG AGC TTC CTG

GAG GTG TCG TAC CGC GTT CTA CGC CAC CTT GCC

CAG CCC

(n is 0 or 1).

10. The method for producing glycoprotein described under Patent Claim 2, in which the gene encoding the polypeptide having a human granulocyte colony-stimulating factor activity has the nucleotide sequence shown in Fig. 3 (A) or a part of it.

11. The method for producing glycoprotein described under Patent Claim 2, in which the gene encoding the polypeptide having a human granulocyte colony-stimulating factor activity has the nucleotide sequence shown in Fig. 4 (A) or a part of it.

12. The method for producing glycoprotein described under Patent Claim 2, in which the human chromosome-derived gene encoding the polypeptide a having human granulocyte colony-stimulating factor activity has the nucleotide sequence shown in Fig. 5 or a part of it.

13. The method for producing glycoprotein described under Patent Claim 2, characterized by the fact that the host cell is an animal cell.

3. Detailed Description

[Field in the Industry]

The present invention is related to a novel glycoprotein having a specific stimulating activity mainly for colonies of human granulocytes, that is, a colony-stimulating factor (abbreviated as CSF in the following), and to a method for producing it.

[Prior Art]

Existence of factors promoting in-vivo formation of colonies has been known through two-layer agar cultures with bone marrow cells, on the upper layer as target cells and with renal or fetal cells on the lower layer, leading to growth and differentiation of the upper layer cells to form colonies from neutrophilic granulocytes (called granulocyte in the following), monocytes or macrophages [Pluznik and Sach: J. Cell. Comp. Physiol., Vol. 66, p. 319 (1965); Bradley and Metcalf: Aust. J. Exp. Biol. Med. Sci., Vol. 44, p. 287 (1966)].

The factors collectively called CSF are known to be produced from normally widely distributed cells such as T cells, monocytes, macrophages, fibroblasts, endothelial cells, etc. Among CSFs, there are subclasses, such as a granulocyte-monocyte/macrophage CSF (abbreviated as GM-CSF) which acts on the stem cells of granulocyte and monocyte/macrophage to stimulate their proliferation and induce their differentiation, forming colonies from granulocytes and monocytes/macrophages in agar, a monocyte/macrophage CSF (abbreviated as M-CSF) which mainly has the effect of forming colonies of monocytes/macrophages, a multi-CSF acting on relatively undifferentiated multiple potential progenitors, and a granulocyte CSF (abbreviated as G-CSF) which mainly forms granulocyte colonies as described in the present invention. It has been considered that the differentiation stages of target cells are different for the various subclasses [Asano: Metabolism and Disease, Vol. 22, p. 249 (1985); Yunis et al., "Growth and Maturation Factors", edited by Guroff, John Willey & Sons, NY, Vol. 1, p. 209 (1983)].

Accordingly, it is critically important for analysis of hematopoietic mechanism and of the pathology of various hematological diseases, to purify each of the subclasses to investigate their chemical properties and biological characteristics in detail.

In particular, the biological effects of G-CSF, induction of the differentiation of bone marrow leukemia cells and enhancement of the functions of mature granulocytes, attract significant attention. Clinical usefulness of G-CSF, particularly for the treatment and prevention of leukemia, is highly expected.

[Problems to be Solved by the Invention]

Traditionally, to isolate and purify G-CSF, cells were cultured and G-CSF was isolated from the culture supernatant. However, G-CSF is produced only at low concentrations. To obtain a small amount of G-CSF from a large quantity of culture liquid, a complicated process of purification is required. Due to this and other problems, purification of homogeneous G-CSF in a large quantity has not been achieved. Accordingly, large scale production of G-CSF using recombinant DNA technology has been desired.

[Means of Solving the Problems]

Against the above background, the inventors succeeded in isolating the gene encoding a polypeptide having the activity of human G-CSF, and also in expressing the gene in host cells.

The present invention is to provide a glycoprotein having a polypeptide with the amino acid sequence described under Patent Claim 1 or a part of it and sugar chains and having the activity of human granulocyte colony-stimulating factor. In addition, the present invention is to provide a method for producing the above glycoprotein. Thus, it is to provide a method for producing the glycoprotein having a human granulocyte colony-stimulating factor activity, characterized by the fact that a gene encoding the polypeptide having human granulocyte colony-stimulating factor activity is obtained, and then a recombinant vector is prepared containing the gene, followed by transformation of the vector in a host cell, culture of the resultant transformant, and isolation of the glycoprotein from the culture.

In the following, the present invention is described in detail.

The gene encoding a polypeptide having human G-CSF activity used in the present invention is a DNA complementary to a messenger RNA (mRNA) that is obtained as a 15-17S faction by sucrose density gradient centrifugation and encodes the polypeptide having the activity of human G-CSF (cDNA).

The inventors obtained two classes of such cDNA. One of the cDNA classes is a gene encoding the polypeptide I or II in Fig. 3 (B) or a part of it. More specifically, it is the sequence from the ATG at nucleotide positions 32-34 to the CCC at nucleotide positions 650-652 or the sequence from the ACC at positions 122-124 to the CCC at positions 650-652 from the 5'-end of the nucleotide sequence shown in Fig. 3 (A), the sequence shown in Fig. 3 (A), or a part of it.

This class of cDNA is called cDNA(+VSE).

The other class of cDNA is a gene encoding the polypeptide I or II in Fig. 4 (B) or a part of it. More specifically, it is the sequence from the ATG at nucleotide positions 31-33 to the CCC at nucleotide positions 640-642 or the sequence from the ACC at positions 121-123 to the CCC at positions 640-642 from the 5'-end of the nucleotide sequence shown in Fig. 4 (A), the sequence shown in Fig. 4 (A), or a part of it.

This class of cDNA is called cDNA(-VSE).

The above genes can be obtained, for example, by preparing the mRNA encoding G-CSF from mammalian cells capable of producing the polypeptide with G-CSF activity, and then converting it into a double-stranded cDNA by the standard method, followed by screening of a recombinant collection containing the DNA (called cDNA library in the following) by a standard method.

The gene used in the present invention includes a gene derived from human chromosomes, which encodes a polypeptide having human G-CSF activity. The human chromosome-derived gene contains nucleotide sequences involved in transcriptional regulation, the nucleotide sequence shown in Fig. 5, or a part of it.

The chromosome-derived gene can be obtained by, for example, preparing a recombinant collection containing the human chromosomal gene from human cells (called human chromosomal gene library in the following) and subsequently screening by a standard method.

In this case, source of the human chromosomal gene can be any human cells, such as cells isolated from liver, kidney or cultured tumor cells, etc.

Preparation of human chromosomal gene library from human cells can be performed by a known method [Maniatis et al.: Cell, Vol. 15, p. 687 (1978) and Maniatis et al.: Molecular Cloning, Cold Spring Harbor Laboratory, p. 269 (1982); etc.]. For example, human chromosomal DNA is extracted with phenol, etc. from human fetal liver, etc. The resultant DNA is digested partially or completely with a restriction enzyme to obtain fragmented DNAs with appropriate lengths. The fragmented DNA is inserted into a λ phage vector DNA fragment using T4 DNA ligase and, if necessary, with a linker containing the cutting site of EcoRI, etc. Then, by in vitro packaging method λ phage plasmid is obtained, which is then transformed into a host cell such as E. coli.

The λ phage used above as vector can be, for example, Charon 4A or EMBL-3, 4, etc.

The above mammalian cells as source of the mRNA in the present invention are the human oral cancer-derived cell line CHU-2 (Collection Nationale de Cultures de Microorganismes (C.N.C.M.) deposition No. I-483) in the present invention. Not only tumor cells, but cells isolated from mammals or other cell lines can also be used.

For the preparation of mRNA, by a method already used in the gene cloning for several physiologically active proteins, such as surface-active agent treatment and phenol treatment in the presence of a ribonuclease inhibitor (e.g. vanadium complex, etc.) [Berger and Birkenmeier: Biochemistry, Vol. 18, p. 5143 (1979)], or guanidine thiocyanate treatment followed by CsCl density gradient centrifugation [Chirgwin et al.: Biochemistry, Vol. 18, p. 5294 (1979)], total RNA is prepared, and then poly(A+)RNA (mRNA) can be obtained by affinity chromatography using oligo(dT)-cellulose or polyU-Sepharose on Sepharose 2B as carrier. Poly(A+)RNA can also be fractionated by sucrose density gradient centrifugation.

To confirm that the mRNA obtained above encodes the polypeptide having G-CSF activity, the mRNA is translated into a protein. The physiological activity is investigated, or the protein is identified using an anti-G-CSF antibody. For example, the mRNA is injected into Xenopus laevis oocytes for translation [Gurdon et al.: Nature, Vol. 233, p. 177 (1972)]. Alternatively, rabbit reticulocyte system or wheat germ system is used for the translation [Schicif and Wensink: "Practical Methods in Molecular Biology", Springer-Verlag, N. Y., (1981)].

For the identification of G-CSF activity, bone marrow cells are used and cultured on agar. Such methods have been reviewed [Metcalf: "Hemapoietic Colonies", Springer-Verlag, Berlin, Heidelberg, NY (1977)].

The mRNA obtained by a method described above is used as template to synthesize a single-stranded cDNA. From this single-stranded cDNA, a double-stranded cDNA is then synthesized, followed by making a recombinant plasmid with an appropriate vector DNA. The plasmid is transformed into (Escherichia coli), etc., thereby obtaining a transformant DNA collection (cDNA library).

For obtaining a double-stranded cDNA from mRNA, for example, oligo(dT) complementary to the polyA chain on the 3'-end of mRNA, is used as primer for reverse transcriptase treatment. Alternatively, an oligonucleotide corresponding to a part of the amino acid sequence of G-CSF is synthesized, and then used as primer for reverse transcriptase treatment to synthesize a cDNA complementary to the mRNA. For obtaining a double-stranded cDNA, after alkaline treatment to degrade and remove mRNA, the resultant single-stranded cDNA is treated with reverse transcriptase or DNA polymerase (e.g. Klenow fragment, etc.) followed by treatment with SI nuclease, etc. Alternatively, it can be obtained by direct treatment with RNase H or DNA polymerase (e.g. DNA polymerase I from E. coli, etc.) [for example, Maniati et al.: Molecular Cloning, Cold Spring Harbor Laboratory (1982); and Gubler and Hoffman: Gene, Vol. 25, p. 263 (1983)].

The double-stranded cDNA thus obtained is recombined into an appropriate vector, such as an EK type plasmid vector (e.g. pSC101, pDF41, ColE1, pMB9, pBR322, pBR327, pACYC1, etc.) or a phage vector (e.g. λgt, λC, λgt10, λgtWES, etc.), followed by transformation into E. coli (x1776, HB101, DH1, C600 strain, etc.), etc., thereby obtaining a cDNA library (for example, see the above "Molecular Cloning").

For ligating the double-stranded cDNA with the vector, an appropriate, chemically synthesized DNA fragment is added to attach a ligatable end to the end of the DNA, and then it is treated along with the vector DNA, that has been opened in advance with a restriction enzyme, with T4 phage DNA ligase in the presence of ATP. Alternatively, dG and dC (or dA and dT) chains are added to the vector DNA, which has been opened in advance with a restriction enzyme, and the double-stranded cDNA, and

then, for example, a solution containing the two DNAs is gradually cooled (see the above "Molecular Cloning").

For transformation of the recombinant DNA thus obtained into a host cell, for example, with E. coli as the host cell, the method described by Hanahan in detail can be used [J. Mol. Biol.: Vol. 166, p. 557 (1983)]. Thus, the recombinant DNA is added to competent cells prepared in the presence of $CaCl_2$, $MgCl_2$ or RbCl for transformation.

To identify cells containing the target gene, one of the following methods can be used: the plus-minus method used for cloning interferon cDNA [Taniguchi et al.: Proc. Jpn. Acad., Vol. 55, Ser. B, p. 464 (1979)], the hybridization-translation assay method [Nagata et al.: Nature, Vol. 284, p. 316 (1980)], the colony or plaque hybridization method using an oligonucleotide chemically synthesized based on the amino acid sequence of the protein as a probe [Wallace et al.: Nucleic Acids Res., Vol. 9, p. 879 (1981) and Benton and Davise: Science, Vol. 196, p. 180 (1977)], etc.

The cloned fragment containing the gene encoding a polypeptide with the activity of human G-CSF thus obtained is recombined into an appropriate vector DNA again, and accordingly it can be transformed into a eukaryotic or prokaryotic host cell. By introducing an appropriate promoter and a sequence(s) for expression, the gene can be expressed in the host cell.

Mammalian animal-derived host cells, for example, include COS cells, Chinese hamster ovary (CHO) cells, C-127 cells, Hela cells, etc. Vectors that can be transformed into these cells include pSV2-gpt [Mulligan and Berg: Proc. Natl. Acad. Sci. USA, Vol. 78, p. 2072 (1981)], etc. Such a vector contains replication initiation, selection marker, and promoter, RNA splice site and polyadenylation signal, etc. upstream the gene to be expressed.

The promoter for gene expression in mammalian cells can be the promoters of retrovirus, polyomavirus, adenovirus, simian virus (SV40), etc. For example, when the promoter of SV40 is used, it can be easily performed by Mulligan et al.'s method [Nature, Vol. 277, p. 108 (1979)].

The replication origin can be one derived from SV40, polyomavirus, adenovirus, bovine papillomavirus (BPV), etc. The selection marker can be phosphotransferase APH

(3') II or I (neo) gene, thymidine kinase (TK) gene, E. coli xanthine-guanine phosphoribosyltransferase (Ecogpt) gene, dihydrofolic acid reductase (DHFR) gene, etc.

To obtain the polypeptide with the activity of human G-CSF using the host-vector system as described above, the recombinant DNA with the gene inserted at an appropriate position of the above vector is transformed into the host cell, followed by culturing of resultant transformant. Known procedures can be used for further isolation and purification of the polypeptide from the cells or culture supernatant,.

In general, eukaryotic genes exhibit polymorphism as exemplified by interferon gene [Nishi et al.: J. Biochem. Vol. 97, p. 153 (1985)]. Due to polymorphism, one or more amino acid residues can be different. There also could be changes of nucleotide sequence without any changes of amino acid residue.

A polypeptide with a deletion or addition of one or more amino acid residues or with one or more amino acid residues substituted by one or more amino acid residues in the amino acid sequence shown in Fig. 3 (B) or Fig. 4 (B) could still have the activity of human G-CSF. For example, it is well-known that a polypeptide, obtained by converting the nucleotide sequence corresponding to cysteine to a nucleotide sequence corresponding to serine in the gene for human interleukin-2 (IL-2), still maintains the activity of interleukin-2 [Wang et al.: Science, Vol. 224, p. 1431 (1984)]. Accordingly, for all polypeptides, native or artificially synthesized, as long as they have the activity of human G-CSF, their glycoproteins obtained by culturing animal cells transformed with recombinant vectors containing a gene encoding such a polypeptide (transformants) are covered by the present invention.

Methods for producing the gene necessary for obtaining the glycoprotein with the activity of human G-CSF, the recombinant vector containing the gene, the transformant containing the vector, and for producing the target glycoprotein by culturing the transformant are described roughly as follows.

(1) Preparation of the probe

The N-terminal amino acid sequence of human CSF purified to homogeneity from the culture supernatant of the tumor cell line CHU-2 was determined. In addition, by bromocyan digestion and trypsin treatment fragments are prepared and their amino acid sequences are also determined [Practical Example 3 (i) (ii) (iii)].

From the amino acid sequences, three nucleotides, probe (A), probe (LC) and probe (IWQ) were synthesized (Practical Example 4).

Probe (A) is a mixed type probe comprising 14 nucleotides. Probe (IWQ) comprises 30 nucleotides using deoxyinosine as used in the gene cloning of human cholecystokinin [Takanishi et al.: Proc. Natl. Acad. Sci. USA, Vol. 82, p. 1931 (1985)].

Probe (LC) is a probe comprising 24 nucleotides synthesized based on the nucleotide sequence shown in Fig. 3, corresponding to the amino acid sequence from No. 32 to No. 39 from the N-terminus shown in Practical Example 3 (i).

Chemical synthesis of nucleotide can be performed by the modified phospho-triester method used as a solid phase method, as reviewed by Marang [Tetrahedron, Vol. 39, p. 3-22 (1983)].

The probes used can be based on amino acid sequences at positions other than those for the probes used in the present invention.

(2) Construction of cDNA library

A guanidine thiocyanate solution is added to CHU-2 cells for homogenization. Total RNA is obtained by the CsCl density gradient centrifugation method.

From the total RNA, poly(A+) RNA is isolated using an oligo(dT) cellulose column. Single-stranded cDNA is synthesized with reverse transcriptase. RNase H and E. coli DNA polymerase are added to obtain double-stranded cDNA. A dC chain is added to the resultant double-stranded cDNA, and the cDNA is connected with pBR322 vector with a dG chain added to the PstI digestion site. By transformation into E. coli strain X1776, a pBR322-based cDNA library is obtained (Practical Examples 5 and 6).

Similarly, a EcoRI linker is used to connect the double-stranded cDNA with λgt10 vector to construct a λ phage-based cDNA library (Practical Example 7).

(3) Screening

Clones of the pBR322-based cDNA library are fixed on Whatman No. 541 filter paper, and ^{32}P-labeled probe (IWQ) is used to perform colony hybridization, thereby selecting one clone. This clone is further investigated by Southern blotting method

[Southern: J. Mol. Biol. Vol. 98, p. 503 (1975)], and it is found that it also hybridizes with probe (A).

The nucleotide sequence of this clone is determined by the dideoxy method [Sanger: Science, Vol. 214, p. 1205 (1981)].

The resultant nucleotide sequence of the cDNA insert is shown in Fig. 2. As shown in Fig. 2, this cDNA insert comprises 308 nucleotides including probe (IWQ) and probe (A). It is thus known that it has an open reading frame encoding a 83-amino acid sequence shown in Practical Example 3 (iii).

The plasmid derived from pBR322 containing the 308 base pairs is abbreviated as pHCS-1 (Practical Example 8).

A DNA fragment containing the 308 base pairs from pHCS-1 is radiolabeled by the nick translation method (see above mentioned "Molecular Cloning"), and used as a probe to perform screening by plaque hybridization [Benton and Davis: Science, Vol. 196, p. 180 (1977)] with the λgt10 phage-based cDNA library, thereby selecting 5 clones. For clones considered to contain the cDNA, their nucleotide sequences are determined as above (Fig. 3 (A)).

As shown in Fig. 3 (A), this cDNA insert has a large open reading frame.

The amino acid sequence encoded by this cDNA can be represented as shown in Fig. 3 (A).

Comparison with the N-terminal amino acid sequence of G-CSF protein shown in Fig. 3(i) reveals that this cDNA contains a nucleotide sequence corresponding to a signal peptide encoded by 90 base pairs starting at the ATG sequence at nucleotide positions 32-34 from the 5'-end and ending at the GCC sequence at positions 119-121, and mature G-CSF polypeptide encoded by 531 base pairs starting at the ACC sequence at positions 122-124 and ending at the CCC sequence at positions 650-652. Thus, the polypeptide of amino acid sequence I shown in Fig. 3(B) comprises 207 amino acid residues, with a molecular weight of 22292.67 dalton. Similarly, the polypeptide of amino acid sequence II comprises 177 amino acid residues, with a molecular weight of 18986.74 dalton (Practical Example 9).

As an initiation site for the protein, in addition to the positions 32-34, ATG at 68-70 are also possible.

The pBR322-maintaining Escherichia coli (E. coli) strain X1776 with the cDNA(+VSE) inserted at the EcoRI digestion site is deposited with the Academy of Industrial Technologies, Institute for Microbiological Industrial Technologies (FERM BP-954).

Fig. 6 shows restriction enzyme digestion sites of the resultant gene.

A plasmid with this cDNA inserted into pBR327 [Soberon et al.: Gene, Vol. 9, p. 287 (1980)] at EcoRI site is called pBRG4.

The pBRG4 thus obtained is digested with the restriction enzyme EcoRI to obtain a DNA fragment including an about 1500 base pair cDNA. The fragment is radiolabeled by the nick translation method (see above mentioned "Molecular Cloning"), and used as a probe to perform screening by plaque hybridization (see above mentioned Benton and Davis's reference) with the λgt10 phage-based cDNA library again. At this time, two nitrocellulose filter papers with the λ phage DNA immobilized are prepared, and plaque hybridization with probe (LC) is similarly performed. Phages positive with both probes are selected, and clones considered to represent full-length are selected. The nucleotide sequences of the cDNA inserts are determined using the dideoxy method, and the results are shown in Fig. 4(A).

This cDNA has a large open reading frame, and encodes an amino acid sequence as shown in Fig. 4(A).

Comparison with the N-terminal amino acid sequence of G-CSF protein shown in Fig. 3(i) reveals that this cDNA contains a nucleotide sequence corresponding to a signal peptide encoded by 90 base pairs starting at the ATG sequence at nucleotide positions 31-33 from the 5'-end and ending at the GCC sequence at positions 118-120, and mature G-CSF polypeptide encoded by 522 base pairs starting at the ACC sequence at positions 121-123 and ending at the CCC sequence at positions 640-642. Thus, the polypeptide of amino acid sequence I shown in Fig. 4(B) comprises 204 amino acid residues, with a molecular weight of 21977.35 dalton. Similarly, the polypeptide of amino acid sequence

II comprises 174 amino acid residues, with a molecular weight of 18671.42 dalton (Practical Example 10).

As an initiation site for the protein, in addition to the positions 31-33, ATGs at 58-60 and 67-69 are also possible.

The pBR327-maintaining Escherichia coli (E. coli) strain X1776 with the cDNA(-VSE) inserted at the EcoRI digestion site is deposited with the Academy of Industrial Technologies, Institute for Microbiological Industrial Technologies (FERM BP-955).

Fig. 6 shows restriction enzyme digestion sites of the resultant gene.

A plasmid with this cDNA inserted into pBR327 at EcoRI site is called pBRV2.

(4) Screening of human chromosomal gene library

A human chromosomal gene library prepared by the method described by Maniatis et al. (the above mentioned Molecular Cloning) was screened with the above-described PHCS-1.

The probe used for the screening can be the 308 base pair DNA fragment derived from PHCS-1, the about 1500 base pair DNA fragment derived from pBRG4, the about 1500 base pair DNA fragment derived from pBRV2, a DNA fragment derived from these DNA fragments with an appropriate length, or the above described oligonucleotide probes IWQ, A or LC.

The PHCS-1-derived DNA fragment was radiolabeled with [^{32}P] by the nick translation method [Roop et al.: Cell, Vol. 15, p. 431 (1978)] and then used as probe to screen the human chromosomal gene library by plaque hybridization (see the above-mentioned Benton and Davis' reference), thereby obtaining a dozen or so clones.

DNA was recovered from the resultant clones, and their restriction enzyme maps were generated by a known method [Fritsch et al.: Cell, Vol. 19, p. 959 (1980)].

Then, the above DNA probe was used to perform Southern blotting (see the Southern reference mentioned above). The experiment revealed that a region encoding

human G-CSF polypeptide existed in an about 4 kbp DNA fragment cut out by EcoRI and XhoI. Accordingly, this about 4 kbp DNA fragment was inserted into pBR327 at the EcoRI site using an EcoRI linker, thereby obtaining pBRCE3β. This plasmid was used to determine the nucleotide sequence of the DNA by the dideoxy method. The nucleotide sequence of about 3 kbp nucleotides was determined, thereby identifying the gene encoding human G-CSF polypeptide (Fig. 5).

E. coli strain x1776 retaining the plasmid pBR327 with this about 4 kbp DNA fragment inserted at the EcoRI site (pBRCE3β), was deposited with the Academy of Industrial Technologies, Institute for Microbiological Industrial Technologies (FERM BP-956).

By comparison between the inserts of pBRG4 and pBRV2 cDNAs shown in Figs. 3 and 4, respectively, it was found that this DNA fragment contains five exons and that it encodes the amino acid sequence deduced from pBRG4 and pBRV2.

This DNA fragment contained not only regions transcribed into human G-CSf mRNA (the chromosomal gene of human G-CSF), but also nucleotide sequences involved in transcriptional regulation [Benoist and Chambon: Nature, Vol. 290, p. 304 (1981) and Breathnack and Chambon: Ann. Rev. Biochem. Vol. 50, p. 349 (1981)].

(5) Construction of recombinant vector for animal cells

Recombinant vectors for C127 cells and NIH3T3 cells as host cells (BPV-derived) and for CHO cells as host cells (including DHFR) were constructed with the +VSE class, -VSE class cDNAs and with the chromosome-derived gene. For COS cells, recombinant vectors were also constructed similarly. Here, typical examples are described, but see practical examples for details.

(A) Construction of +VSE class recombinant vectors

The cDNA (+VSE) fragment obtained in above (3) was ligated with the vector pdKCR to obtain the plasmid pHGA410 (Practical Example 12) (Fig. 8). This plasmid was partially digested with EcoRI, and the terminus was converted to a blunt end. A HindIII linker was added to this DNA, and then HindIII treatment was performed, followed by T4 DNA ligase treatment. The rubidium chloride method (see above-mentioned Molecular Cloning) was used to perform transformation in E. coli strain DHI. The resultant plasmid was named pHGA410 (H) (Fig. 9).

PHGA410 (H) was digested with SalI, and the terminus was converted to a blunt end. After HindIII treatment, the HindIII-SalI fragment was recovered.

On the other hand, the pdBPV-1 plasmid containing the transformation fragment of bovine papilloma virus was treated with HindIII and PvuII. The bigger DNA fragment was isolated and then ligated with the above HindIII-SalI fragment. After transformation in E. coli strain DHI, a pHGG4-derived, CSF cDNA-containing plasmid (pTN-G4) was obtained (Fig. 9) (Practical Example 13).

On the other hand, pHGA410 plasmid or pHGA410 (H) plasmid and pAdD26SVpA plasmid were used to construct a recombinant vector (+VSE) (pHGG4-dhfr) for CHO cells (Figs. 10a and b) (Practical Example 15).

Furthermore, from pAdD26SVpA a 2 kb DNA fragment containing DHFR gene was recovered by treatment with EcoRI and BamHI, and inserted into pHGA410 (H) at the HindIII site, thereby constructing pG4DR1 and pG4DR2 (Fig. 10c) (Practical Example 15).

(B) Construction of -VSE class recombinant vectors

The cDNA (-VSE) fragment obtained in above (3) was ligated with the vector pdKCR to obtain the plasmid pHGV2 (Practical Example 18). This plasmid was partially digested with EcoRI, and the terminus was converted to a blunt end. A HindIII linker was added to this DNA, and then HindIII treatment was performed, followed by T4 DNA ligase treatment. The rubidium chloride method (see the above-mentioned Molecular Cloning) was used to perform transformation in E. coli strain DHI. The resultant plasmid was named pHGV2 (H) (Fig. 12).

PHGV2 (H) was digested with SalI, and the terminus was converted to a blunt end. After HindIII treatment, the HindIII-SalI fragment was recovered. On the other hand, pdBPV-1 plasmid containing the transformation fragment of bovine papilloma virus was treated with HindIII and PvuII. The bigger DNA fragment was isolated and then ligated with the above HindIII-SalI fragment. After transformation in E. coli strain DHI, a pHGV2-derived, CSF cDNA-containing plasmid (pTN-V2) was obtained (Fig. 12) (Practical Example 19).

As with +VSE, pHGV2 plasmid or pHGV2 (H) plasmid and pAdD26SVpA plasmid were used to construct a recombinant vector (-VSE) (pHGV2-dhfr) for CHO cells (Figs. 13a and b) (Practical Example 21).

Furthermore, from pAdD26SpA a 2 kb DNA fragment containing DHFR gene was recovered by treatment with EcoRI and BamHI, and inserted into pHGV2 (H) at the HindIII site, thereby constructing pV2DR1 and pV2DR2 (Fig. 13c) (Practical Example 21).

(C) Construction of recombinant vectors containing chromosome-derived gene

The plasmid pBRCE3β containing the chromosomal gene shown in Fig. 5 obtained in above (4) was digested with EcoRI.

On the other hand, the $pSVH^+K^+$ plasmid described by Bancrji et al. [Cell, Vol. 27, p. 299 (1981)] was treated with KpnI to remove the globulin gene. Furthermore it was partially digested with HindIII to remove a part of late phase genes of SV40. After re-ligation, the expression vector pML-E^+ was obtained.

This vector was treated with the restriction enzyme EcoRI and then dephosphorylated with alkaline phosphatase (Takara Syuzo). The resultant vector DNA was ligated with the above chromosomal DNA fragment using T4 DNA ligase (Takara Syuzo), thereby obtaining the recombinant vector pMLCE3α for COS cells (Practical Example 24). As shown in Fig. 14, this plasmid is one containing the enhancer of the SV40 gene, the replication initiation region of SV40, the replication initiation region of the pBR322 and β-lactamase gene derived from pBR322 (Amp^r). The chromosomal gene of human G-CSF was ligated downstream of the enhancer of the SV40 gene. Construction of the recombinant vector for C127 cells was performed as follows.

Thus, a DNA fragment containing the chromosomal gene of human CSF was cut out with restriction enzymes from the recombinant vector pMLCE3α for COS cells, and then ligated with a DNA fragment containing the replication initiation of bovine papilloma virus (BPV) and a DNA fragment having the initial promoter of SV40 using the T4 DNA ligase. In the resultant pTNCE3α, the chromosome-derived CSF gene was ligated downstream of the initial promoter of SV40. The vector contained 65% of BPV.

On the other hand, as above, the expression vector for CHO cells was obtained by ligating the chromosome-derived CSF gene, the DNA fragment having the initial promoter of SV40 and the pAdD26SVpA-derived DNA fragment containing DHFR gene with T4 DNA ligase. In the resultant pT26SVCE3α, the chromosome-derived CSF gene was ligated downstream of the SV40 promoter, while the DHFR gene was downstream of the major late phase promoter of the adenovirus.

(6) Transformation in animal cells

Here, representative examples are described. See appropriate practical examples for other examples.

(A) Transformation in mouse C127 cells

pTN-G4 plasmid or pTN-V2 plasmid was treated with BamHI in advance, and then transformed into C127 cells that had been cultured to grow, using the phosphoric acid-calcium method. The resultant transformed cells were cultured and clones of high CSF production were selected.

The expressed G-CSF was recovered and purified from the culture supernatant of the transformed cells. The activity of human G-CSF was confirmed. The target glycoprotein was also confirmed by amino acid analysis and sugar chain content analysis of the purified sample.

Regarding sugar chain content analysis, the CSF sample used for amino acid analysis was subjected to amino sugar quantification by the Elson-Margan method, neutral sugar quantification by the orcinol sulfuric acid method or sialic acid quantification by the thiobarbital method.

The quantitative analyses are described in the Biochemical Experiment Series, Vol. 4 "Carbohydrate Chemistry (Second Part), Chapter 13 (Tokyo Kagaku Dojin). After conversion of the quantitative values to weight%, the carbohydrate content of the resultant G-CSF varied in a range of 1-20 (wt%) depending on expression vector and culture conditions.

(B) Transformation in COS cells

The vector pMLCE3α containing the human chromosome-derived G-CSF gene obtained in above (5)-(C) was transfected into COS cells as host cells, that are derived

from monkey CV-1 cells with the replication origin of SV40 deleted and express the transformed large T antigen of SV40 [Gluzman et al.: Cell Vol. 32, p. 175 (1981)], and consequently the activity of human G-CSF was exhibited (Practical Example 25).

Furthermore, the COS cells were collected and subjected to mRNA analysis. The results showed the presence of mRNAs corresponding to the amino acid sequences shown in Fig. 3 (A) and Fig. 4 (A).

[Practical Examples]

In the following, practical examples are used to further describe the present invention in detail. First, the assay for CSF activity is described as a reference example.

<Reference Example>Assay for CSF activity

In the present invention, assay for CSF activity (abbreviated as CSA in the following) is performed as follows.

[Assay for CSA]

(a) Use of human bone marrow cells

It is performed by the single layer soft agar culture method in accordance with Bradley T. R., Metcalf D. et al.'s method [Aust. J. Exp. Biol. Med. Sci. Vol. 44, p. 287-300 (1966)]. Thus, 0.2 mL of bovine fetal serum, 0.1 mL of test sample, 0.1 mL of non-adherent human bone marrow cell suspension (1-2 x 10^5 nucleated cells), 0.2 mL of modified McCoy's 5A culture medium, and 0.4 mL of modified McCoy's 5A culture medium containing 0.75% agar are mixed and transferred into a plastic tissue culture dish with a diameter of 35 mm for solidification, followed by culture at 37°C under conditions of 5% carbon dioxide/95% air and 100% humidity. After 10 days, the formed colonies are counted (a collection of at least 50 cells is counted as one colony). Activity forming one colony is defined as one unit, thereby calculating CSA.

(b) Use of mouse bone marrow cells

0.4 mL of horse serum, 0.1 mL of test sample, 0.1 mL of C3H/Hc (female) mouse bone marrow cell suspension (0.5-1 x 10^5 nucleated cells), and 0.4 mL of modified McCoy's 5A culture medium containing 0.75% agar are mixed and transferred into a plastic tissue culture dish with a diameter of 35 mm for solidification, followed by culture at 37°C under conditions of 5% carbon dioxide/95% air and 100% humidity for 5 days. The formed colonies are counted (one collection of at least 50 cells is counted as one

colony). Activity forming one colony is defined as one unit, thereby calculating the CSA.

The modified McCoy's 5A culture medium used in above methods (a) and (b) and the non-adherent human bone marrow cell suspension used in above method (a) are prepared as follows.

[Modified McCoy's 5A culture medium (2x concentration)]

12 g of McCoy's 5A culture medium (GIBCO), 2.55 g of MEM amino acid vitamin medium (Nissui Pharmaceuticals), 2.18 g of sodium bicarbonate and 50000 units of potassium penicillin G are dissolved in 500 mL of double-distilled water, followed by filtration through a 0.22 μm Millipore filter.

[Non-adherent human bone marrow cell suspension]

Bone marrow fluid from healthy donors obtained by chest bone puncture is diluted 5 times with RPMI 1640 culture medium, and then layered onto Ficol-Paque liquid (Pharmacia). After centrifugation at 400 x g for 30 min at 25°C, cells at the interface are recovered (specific gravity <1.077). The cells are washed, and suspended in RPMI 1640 medium containing 20% fetal bovine serum at a density of 5 x 10^6 cells/mL, followed by transfer to a 25 cm^2 plastic tissue culture flask. After culture in a carbon dioxide incubator for 30 min, non-adherent cells in the supernatant are recovered, and transferred to a 25 cm^2 plastic tissue culture flask again. After culture for 2 hr and 30 min, non-adherent cells in the supernatant are collected for use.

Practical Example 1: Establishment of CHU-2

The tumor of an oral cancer patient with a significant neutrophilia was transplanted into a nu/nu mouse. This tumor showed significant growth and an increased neutrophil count about 10 days after transplantation. The tumor was isolated under sterile conditions 12 days after transplantation, and cut into 1-2 mm^3 cubes, followed by culture as follows.

Then, 10-15 pieces of the tumor cut above were placed in a 50 mL plastic centrifuge tube, and 5 mL of a trypsin solution (containing 0.25% trypsin and 0.02% EDTA) was added. After 10 minutes of shaking in a 37°C water bath, the supernatant was discarded. Again, 5 mL of the same trypsin solution was added, and trypsin treatment was performed at 37°C for 15 min with agitation.

The cell suspension as the supernatant was recovered, and 1 mL of bovine fetal serum was added to stop the action of trypsin, followed by storage on ice.

The above procedures were repeated to recover the cell suspension, which was then combined with the one obtained previously. By centrifugation at 1,500 rpm for 10 min, a cell pellet was obtained.

The cell pellet was washed twice with F-10 containing 10% fetal bovine serum, and then seeded in a 25 cm^2 plastic tissue culture flask at a concentration of 5 x 10^6 cells/flask. An F-10 culture medium containing 10% fetal bovine serum was used. After overnight incubation in a carbon dioxide incubator (carbon dioxide concentration: 5%; humidity: 100%), the supernatant and non-adherent cells were removed. Fresh culture medium was added, and the culture was continued. On day 6 from the start of culture, the cells grew into confluence. At that point the culture medium was replaced with a fresh one. On next day, the culture medium was discarded, and 2 mL of anti-mouse erythrocyte antibody (Cappel) diluted 5-fold with RPMI 1640 and 2 mL of guinea pig complement (Kyokuto Pharmaceuticals) diluted 2.5-fold with the same RPMI 1640 were added, followed by culture at 37°C for 20 min. After incubation, the fibroblasts derived from nu/nu mouse were removed by washing twice with F-10 containing 10% fetal bovine serum. F-10 culture medium containing 10% fetal bovine serum was added, followed by further culture for 2 days. An aliquot of the cells was removed for cloning by the limiting dilution method. Eleven clones were obtained, and their CSF activity was investigated. A clone with an about 10-fold higher activity than others was obtained (CHU-2).

Practical Example 2: Isolation of CSF

Cells were collected from two 150 cm^3 culture flasks with the cells established as above grown into complete confluence. The cells were suspended in 500 mL of F-10 culture medium containing 10% fetal bovine serum, and then transferred to a 1580 cm^3 glass roller bottle (Belco), followed by rotary culture at a speed of 0.5 rpm. When the cells grew to complete confluence on the inner wall of the roller bottle, the culture medium was replaced with serum-free RPMI 1640. After culture for 4 days, the culture supernatant was collected. F-10 culture medium containing 10% fetal bovine serum was added, and the culture was continued. After culture for 3 days, again the culture medium was replaced with serum-free RPMI 1640. After culture for 4 days, the culture

supernatant was collected. By repeating the same procedures, every week 500 mL of serum-free culture medium was obtained from one bottle. Moreover, by this method, the cells could be maintained over a fairly long period of time, and the culture supernatant could be collected. To 5 L of the resultant culture supernatant as one batch, Tween 20 was added to 0.01%. After concentration about 1000-fold by ultrafiltration using Hollow Fiber DC-4 and Amicon PM-10 (Acon), purification was performed by the following procedures.

(i) An Ultrogel ACA 54 column (LKB) with a diameter of 4.6 cm and a length of 90 cm was used. Gel filtration of 5 mL of the above culture supernatant was performed with 0.01 M Tris-hydrochloric acid buffer (pH 7.4) containing 0.15 M NaCl and 0.01% Tween 20 (Kanai Chemicals) at a flow rate of about 50 mL/hr. The column was subjected to calibration in advance with bovine serum albumin (molecular weight 67,000), ovalbumin (molecular weight 45,000), and cytochrome C (molecular weight 12,400). After gel filtration, 0.1 mL of each fraction was used, after 10-fold dilution, for activity assay as described above in "CSA assay method (b)". The result revealed that there was a macrophage-preferred CSA in fractions of Ve = 400-700 mL, and a granulocyte-preferred CSA in fractions of Ve = 800-1200 mL. The later fractions were pooled, and concentrated to about 5 mL by ultrafiltration using PM-10 (Amicon).

(ii) To the above concentrated sample, 0.1% trifluoroacetic acid solution containing 30% n-propanol (Tokyo Kassei, amino acid sequencing grade) was added. After allowing to stand on ice for 15 min, centrifugation was performed at 15,000 rpm for 10 min to remove the precipitate. Subsequently, after application to a μ Bondapak C18 column (Waters, for semi-preparation, 8 mm x 30 cm) that had been equilibrated with the above n-propanol/trifluoroacetic acid solution, elution was performed with a 30-60% linear concentration gradient of n-propanol-containing 0.1% trifluoroacetic acid solution. The high performance liquid chromatography apparatus was Hitachi model 685-50, with the detector being Hitachi model 638-41 detector (both from Hitachi Production). Absorption at both 220 nm and 280 nm was measured. After elution, 10 μL of each fraction was used, after 100-fold dilution, for activity assay as described above in "CSA assay method (b)". The results revealed that there was a peak eluted at 40% of n-propanol. The peak was pooled and re-chromatographed under the same conditions, followed by CSA assay in the same way. Again, the activity peak was observed at 40% of n-propanol. This peak was pooled (4 fractions = 4 mL) and freeze-dried.

(iii) The above freeze-dried powder was dissolved in 200 μl of 0.1% trifluoroacetic acid solution containing 40% n-propanol, and then applied to high performance liquid chromatography (HPLC) using a TSK-G3000SW column (Toyo Sotatsu, 7.5 mm x 60 cm). Elution was performed with the same solution at 0.4 mL/min, and fractions of 0.4 mL each were collected with a fraction collector of FRAC-100 (Pharmacia). The fractions were investigated for CSA as above. The activity was observed in a fraction with a retention time of 37-38 min (the molecular weight corresponds to about 20,000). This fraction was recovered, and further purified on an analytical μ Bondapak C18 column (4.6 mm x 30 cm). The main peak was recovered and freeze-dried. The resultant sample was evaluated by the above "CSA assay method (a)", and it was confirmed to have the activity of human G-CSF.

Practical Example 3: Determination of amino acid sequence

(i) Determination of N-terminal amino acid sequence

Using a gas phase sequencer (Applied Biosystems), the test sample was subjected to Edman degradation, and then the resultant PTH amino acid was analyzed by a standard method using a high performance liquid chromatography apparatus (Beckman Instruments)) and an Ultrasphere-ODS column (Beckman Instruments)). The column (5 μm, diameter 4.6 mm, length 250 cm) was equilibrated with a starting buffer (15 mM sodium acetate buffer, pH 4.5, containing 40% acetonitrile), and then the test sample (dissolved in 20 μL of the starting buffer) was applied. Isocratic elution was performed with the starting buffer at a flow rate of 1.4 mL/min. The column temperature was maintained at 40°C. Detection of PTH amino acid was performed based on ultraviolet absorption at 269 nm and 320 nm. Standard PTH amino acids (Sigma), 2 nmol each, were separated in advance by the same system to determine their retention times. The test sample it could be identified based on the retention time.

The results revealed that the amino acid sequence from the N-terminus to No. 40 residue was as follows.

H_2 N-Thr-Pro-Leu-Gly-
Pro-Ala-Ser-Ser-Leu-
Pro-Gln-Ser-Phe-Leu-
Leu-Lys-Cys-Leu-Glu-
Gln-Val-Arg-Lys-Ile-
Gln-Gly-Asp-Gly-Ala-
Ala-Leu-Gln-Glu-Lys-
Leu-Cys-Ala-Thr-Tyr-
Lys-

(ii) Bromocyan degradation

The test sample was dissolved in 70% formic acid, and 200 equivalents of sublimation-purified bromocyan was added, followed by reaction overnight at 37°C. The reaction mixture was freeze-dried, and fractionated by HPLC on a TSK G3000SW column (Toyo Sotatsu), thereby obtaining four peaks, named CN-1, CN-2, CN-3 and CN-4 in the order of molecular weights, from large to small. CN-1 and CN-2, the yields of which were higher, were analyzed using a gas phase sequencer (Applied Biosystems) under the same conditions as in (i).

The results revealed that CN-1 was a peptide from the N-terminus of G-CSF, and that CN-2 had the following amino acid sequence.

Pro-Ala-Phe-Ala-Ser-
Ala-Phe-Gln-Arg-Arg-
Ala-Gly-Gly-Val-Leu-
Val-Ala-Ser-His-Leu-
Gln-

(iii) Trypsin degradation

The test sample was dissolved in 0.1 M Tris-hydrochloric acid buffer (pH 7.4) containing 8M urea, and then 0.1 M Tris-hydrochloric acid buffer (pH 7.4) containing 0.1% 2-mercaptoethanol was added so that the final concentration of urea was 2 M. Subsequently, TPCK-treated trypsin (Sigma) was added so that the ratio of test sample to enzyme was 50 :1. After reaction at 25°C for 4 hr, the same amount of TPCK-treated trypsin was further added, followed by further reaction at 25°C for 16 hr. After reaction, the reaction mixture was subjected to reverse phase high performance liquid chromatography using a C8 column (Yamamura Chemical). For elution, n-propanol containing 0.1% TFA was used and the concentration of n-propanol was increased linearly between 5% and 60%. Among the peaks detected by ultraviolet absorption at 280 nm, the main peak was subjected to amino acid sequencing using a gas phase sequencer (Applied Biosystems) under the same conditions as in (i). The results revealed that the main peak present had the following sequence, which contained a portion of CN-2 of (ii).

Gln-Leu-Asp-Val-Ala-
Asp-Phe-Ala-Thr-Thr-
Ile-Trp-Gln-Gln-Met-
Glu-Glu-Leu-Gly-Met-
Ala-Pro-Ala-Leu-Gln-
Pro-Thr-Gln-Gly-Ala-
Met-Pro-Ala-Phe-Ala-
Ser-

Practical Example 4: Preparation of DNA probe

(i) Synthesis of probe (IWQ)

Based on the 10 amino acid residue sequence 5Ile-Trp-Gln-Gln-Met-Glu-Glu-Leu-Gly-Met among the amino acid sequences obtained in Practical Example 3 (iii), a 30-mer nucleotide sequence was obtained (Fig. 1). In the sequence shown in Fig. 1, for example, at the No. 9 position from 5'-end, it is an equal amount mixture of dA and dG. Raw material nucleotides used were mainly dimers. If necessary, mononucleotides can also be used. 20 mg of starting material nucleotide resin Ap-d(G) (Yamasa Soybean

Sauce) was applied to a column equipped with glass filter, and thoroughly washed with methylene chloride. The 4, 4'-dimethoxytrityl group was removed with methylene chloride solution containing 3% trichloroacetic acid. Subsequently, the column was washed several times with 1 mL of methylene chloride, and then washed with anhydrous pyridine to replace the solvent. 20 mg of nucleotide dimer (DMTr)ApTp(NHR_3) (Nippon Zeon; NHR_3 is triethylammonium, while DMTr is dimethoxytrityl) and 0.2 mL pyridine were added. The inside of the column was vacuum-dried with a vacuum pump. 20 mg of 2, 4, 6-trimethylbenzenesulfonyl-3-nitrotriazolide (MSNT, Wako Pure Chemicals) and 0.2 mL of anhydrous pyridine were added. The air inside of the column was replaced with nitrogen gas. By shaking at room temperature for 45 min, the nucleotide resin and dimer were condensed. After reaction, the column was washed with pyridine, and then the unreacted OH groups were acetylated with an excess amount of acetic acid anhydride/4-dimethylaminopyridine. The column was washed with pyridine again. Subsequently, the same procedures were repeated to condense the following in this particular order: (DMTr)Ip(NHR_3), (DMTr)GpGp(NHR_3), (DMTr)Ip(NHR_3), equal amount mixture of (DMTr)CpTp(NHR_3) and (DMTr)TpTp(NHR_3), equal amount mixture of (DMTr)ApAp(NHR_3) and (DMTr)ApGp(NHR_3), equal amount mixture of (DMTr)ApGp(NHR_3) and (DMTr)GpGp(NHR_3), (DMTr)GpAp(NHR_3), (DMTr)TpGp(NHR_3), equal amount mixture of (DMTr)ApAp(NHR_3) and (DMTr)GpAp(NHR_3), (DMTr)CpAp(NHR_3), equal amount mixture of (DMTr)ApAp(NHR_3) and (DMTr)ApGp(NHR_3), (DMTr)GpCp(NHR_3), (DMTr)TpGp(NHR_3), (DMTr)Ip(NHR_3) and (DMTr)ApTp(NHR_3) [(DMTr)Ip(NHR_3) was from Yamasa Soybean Sauce, while others were from Nippon Zeon]. After the final reaction step, without acetylation, the resin was washed with pyridine, methylene chloride and ether, in this particular order, followed by drying. The dried resin was suspended in 1.7 mL of a mixture of 1 mL of dioxane containing 1 M tetramethylguanidine and 1 M α-picolinic aldoxime, 0.5 mL of pyridine and 0.2 mL of water. After allowing to stand at room temperature overnight, it was concentrated under a reduced pressure until 100-200 μL. A small amount of pyridine (2-3 drops) was added to the concentrate and then 2-3 mL of concentrated ammonia water was added, followed by heating at 55°C for 6 hr. Ethyl acetate was added for extraction and separation. The resultant aqueous phase was concentrated under a reduced pressure, and then dissolved in 50 mM triethylammonium acetate solution (pH 7.0), followed by column chromatography using a C-18 column (1.0 x 15 cm, Waters). Elution was performed with a 10%-30% linear concentration gradient of acetonitrile in 50 mM

triethylammonium acetate buffer (pH 7.0). A peak eluted at an acetonitrile concentration of around 25% was concentrated under reduced pressure.

To the concentrate, 80% acetic acid solution was added, and the mixture was allowed to stand at room temperature for 30 min. Ethyl acetate was added for extraction and separation. The aqueous phase was concentrated under reduced pressure. The resultant concentrate was applied to high performance liquid chromatography using a C-18 column (Sensyu Scientific, SSC-ODS-272, 6 ø x 200 mm) for further purification. Elution was performed with a 10%-20% linear concentration gradient of acetonitrile in 50 mM triethylammonium acetate buffer (pH 7.0). The synthetic DNA was obtained at a yield of at least 10 A_{260} units.

(ii) Synthesis of probe (A)

Based on the 5 amino acid residue sequence Met-Pro-Ala-Phe-Ala among the amino acid sequences obtained in Practical Example 3 (iii), a 14-mer nucleotide sequence was obtained (Fig. 1).

The synthesis was performed by the same method as with probe (IWQ). To nucleotide resin Ap-d(T) (Yamasa Soybean Sauce), (DMTr)CpAp(NHR_3), (DMTr)GpGp(NHR_3), (DMTr)CpAp(NHR_3), (DMTr)CpTp(NHR_3), equal amount mixture of (DMTr)CpGp(NHR_3) and (DMTr)CpCp(NHR_3), (DMTr)ApGp(NHR_3), (DMTr)TpGp(NHR_3), equal amount mixture of (DMTr)GpGp(NHR_3) and (DMTr)CpGp(NHR_3), (DMTr)ApAp(NHR_3), equal amount mixture of (DMTr)CpAp(NHR_3) and (DMTr)CpGp(NHR_3), and (DMTr)Gp(NHR_3) (all from Nippon Zeon) were condensed sequentially, in this particular order, thereby the synthetic DNA at a yield of at least 10 A_{260} units was obtained.

The sequence of the resultant oligonucleotide was determined by the Maxam-Gilbert method, and it was confirmed to have the sequence shown in Fig. 1.

(iii) Synthesis of probe (LC)

Automated synthesis was performed using DNA synthesizer model 380A from Applied Biosystems. This method was based on the principles described by Caruthers et al. [J. Am. Chem. Soc. Vol. 103, p. 3185 (1981)], and is called the phosphamidite method.

The phosphoamidite form of (DMTr)-dT activated with tetrazole in advance was condensed to dG-S (S is a carrier) with the 5'-dimethoxytrityl group (DMTr) de-protected. The unreacted hydroxyl groups were acetylated, and then in the presence of water iodic acid treatment was performed to introduce a phosphate group. DMTr group was de-protected, and then the same procedures were repeated to synthesize the 24-mer nucleotide with the sequence shown in Fig. 1. The resultant nucleotide was cleaved from the carrier and de-protected, followed by purification by reverse phase high performance liquid chromatography using a C18 column (Sensyu Scientific, SSC-ODS-272).

Practical Example 5: Culture of CHU-2 cells and purification of mRNA

1) Culture of CHU-2 cells and recovery of cells

The established CHU-2 cells were grown to complete confluence in two 150 cm^2 culture flasks. The cells were suspended in 500 mL of RPMI 1640 culture medium containing 10% fetal bovine serum, and then transferred to a 1580 cm^3 glass roller bottle (Belco). After rotary culture for 4 days at a speed of 0.5 rpm, when the cells grew into complete confluence on the inside wall of the roller bottle, the culture medium was discarded from the roller bottle. 100 mL of physiological saline containing 0.02% EDTA that had been heated to 37°C in advance was added. After heating at 37°C for 2 min, the cells were removed from the wall by pipeting. The resultant cell suspension was centrifuged at 1500 rpm for 10 min to obtain a cell pellet. The cells were suspended in 5 mL of physiological saline without containing EDTA, and centrifuged at 1500 rpm for 10 min to obtain a cell pellet again (wet weight about 0.8 g). The cells thus obtained were stored at -80°C until RNA isolation.

2) Purification of mRNA

mRNA isolation from the CHU-2 cells obtained above was essentially performed as described in Molecular Cloning [Maniatis et al.: Cold Spring Harbor, p. 196 (1982)]. The CHU-2 cells stored frozen (wet weight 3.8 g) were suspended in 20 mL of 6 M guanidine solution (6 M guanidine cyanate, 5 mM sodium citrate (pH 7.0), 0.1 M β-mercaptoethanol, and 0.5% sodium sarcosylsulfate), and thoroughly mixed for 2-3 min with a vortex mixer. In a 20 mL syringe equipped with an 18 G injection needle, the mixture was aspirated and discharged 10 times. 6 mL of 5.7 M CsCl - 0.1 M EDTA (pH 7.5) was first placed on a polyallomer centrifugation tube that fits into the Beckman SW40Ti rotor, and then about 6 mL of the above viscous cell homogenate in guanidine solution was introduced so that the tube was full. Four tubes such prepared were

centrifuged at 30,000 rpm for 15 hr at 20°C. The resultant pellets were washed three times with a small amount of 70% ethanol.

The pellets from all tubes were pooled and dissolved in 550 μL of water. The NaCl concentration was adjusted to 0.2 M. After phenol-chloroform (1:1) treatment and chloroform treatment, 2.5 volumes of ethanol were added to perform ethanol precipitation, thereby obtaining the total RNA (from 3.8 g of wet cells about 10.1 mg of total RNA was obtained).

Poly(A^+) RNA purification from total RNA was performed as follows. The method was affinity purification, based on the fact that on the 3'-end of mRNA there is a polyA chain. Oligo(dT)-cellulose (P-L Biochemicals, Type 7) was used. The total RNA was dissolved in an adsorption buffer (10 mM Tris-hydrochloric acid (pH 7.5) containing 0.5 M NaCl, 1 mM EDTA, and 0.1% SDS). After heating at 65°C for 5 min, the solution was applied onto an oligo(dT)-cellulose column that had been filled with the same buffer. Elution was performed with TE buffer (10 mM Tris-hydrochloric acid (pH 7.5) containing 1 mM EDTA). The flow-through fraction was applied to the column again for the same operation, and combined with the sample from the first operation. Using these procedures, 400 μg of poly(A^+) RNA was obtained.

The mRNA thus obtained was subjected to size fractionation by sucrose density gradient centrifugation as described in Schicif and Wensink's book on experimental techniques [Practical Methods in Molecular Biology, Springer-Verlag, New York, Heidelberg, Berlin (1981)].

Thus, in the tube for SW40Ti rotor (Beckman), a 5%-25% sucrose density gradient was prepared. The sucrose solutions were at 5% and 25% of sucrose (RNase-free, from Schwarz/Mann) in 10 mM Tris-hydrochloric acid (pH 7.5) containing 0.1 M NaCl, 1 mM EDTA and 0.5% SDS.

800 μg of the mRNA (poly(A^+) RNA) prepared above was dissolved in 200 μL – 500 μL of TE buffer. After heating at 65°C for 5 min, the solution was cooled quickly and then layered on the sucrose density gradient. After centrifugation at 30,000 rpm for 20 hr, fractions of 0.5 mL each were collected and the absorbance at 260 nm was measured. From standard RNAs (28S, 18S and 5S ribosome RNAs) subjected to the same procedures, the sizes of the fractionated RNA were determined. Moreover, the G-

CSF activity of each fraction was investigated using the Xenopus laevis oocyte system. Thus, mRNA from each fraction was prepared as 1 μg/μL in water, and then injected into oocytes isolated from Xenopus laevis (about one year old) at 50 ng mRNA/cell. The oocytes were placed in 96-well microtiter plates at 10 cells/well. 100 μL of culture medium (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 7.5 mM Tris-hydrochloric acid (pH 7.6), 10 mg/L of penicillin and 10 mg/L of streptomycin) was added to each well. After culture at room temperature for 48 hr, the supernatant was recovered. After concentration and purification, the G-CSF activity was assayed.

The results revealed G-CSF activity in the 15-17S fractions.

Practical Example 6: Synthesis of cDNA (construction of pBR-based cDNA library)

From the poly(A^+) RNA prepared above, cDNA was obtained by Land et al.'s method [Nucleic Acids Res. Vol. 9, p. 2251 (1981)] with modifications from Gubier and Hoffman's method [Gene, Vol. 25, p. 263 (1983)].

1) Synthesis of single-stranded cDNA

In an Eppendorf 1.5 mL tube, reagents were added in the following order: 80 μL of reaction buffer (500 mM KCl, 50 mM $MgCl_2$, 250 mM Tris-hydrochloric acid, pH 8.3), 20 μL of 200 mM dithiothreitol, 32 μL of 12.5 mM dNTP (dATP, dGTP, dCTP and dTTP each at 12.5 mM), 10 μL of α-^{32}P-dCTP (Amersham, PB 10205), 32 μL of oligo$(dT)_{12-18}$ (P-L Biochemicals, 500 μg/mL), 20 μL of poly(A^+)-RNA (2.1 μg/μL), and 206 μL of distilled water (a total of 400 μL of the reaction mixture). After heating at 65°C for 5 min, the mixture was maintained at 42°C for 5 min. To this reaction mixture, 120 units of a reverse transcriptase (Takara Syuzo) was added, followed by further reaction at 42°C for 2 hr. 2 μL of RNase inhibitor (Bethesda Research Laboratories), 20 μL of TE buffer, 16 μL of 100 mM sodium pyrophosphate and 48 units (4 μL) of the reverse transcriptase were further added, and then further reaction was performed at 46°C for 2 hr. 8 μL of 0.5 M EDTA and 8 μL of 10% SDS were added to stop the reaction. After phenol-chloroform treatment and ethanol precipitation (twice), a single-stranded cDNA was obtained.

2) dC addition to single-stranded cDNA

The single-stranded cDNA obtained above was dissolved in 60 μL of distilled water, and then 60 μL of a dC chain addition buffer (400 mM potassium cacodylate, 50 mM Tris-hydrochloric acid (pH 6.9), 4 mM dithiothreitol, 1 mM $COCl_2$, and 1 mM dCTP) was added. After heating at 37°C for 5 min, 3 μL of a terminal transferase (27 units/μL, P-L Biochemicals) was added, followed by reaction at 37°C for 2.5 min. After phenol-chloroform treatment (once) and ethanol precipitation (twice), the pellet was dissolved in 40 μL of TE buffer containing 100 mM NaCl.

3) Synthesis of double-stranded cDNA

To the above 40 μL of cDNA solution, 4 μL of oligo$(dG)_{12-18}$ (200 μg/mL, P-L Biochemicals) was added, followed by heating at 65°C for 5 min and then at 42°C for 30 min. The reaction mixture was maintained at 0°C. To this reaction mixture, 80 μL of buffer (100 mM Tris-hydrochloric acid, pH 7.5, 20 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$, 500 mM KCl), 4 μL of 4 mM dNTP (dATP, dGTP, dCTP and dTTP each at 4 mM), 60 μL 1mM ß-NAD, 210 μL of distilled water, 20 μL of E. coli DNA polymerase I (Takara Syuzo), 15 μL of E. coli DNA ligase (Takara Syuzo), and 15 μL of E. coli RNase H (Takara Syuzo) were added. After reaction at 12°C for 1 hr, 4 μL of 4 mM dNTP was further added, followed by further reaction at 25°C for 1 hr. After phenol-chloroform treatment and ethanol precipitation (once), about 8 μg of double-stranded cDNA was obtained. This double-stranded cDNA was dissolved in TE buffer and subjected to 1.2% agarose gel electrophoresis. Fractions corresponding to about 560 base pairs (bp) to 2 k base pairs (kbp) were eluted by adsorption on a Whatman DE81 filter paper (Whatman), thereby obtaining about 0.2 μg.

4) dC addition to double-stranded cDNA

The double-stranded cDNA obtained above was dissolved in 40 μL of TE buffer, and then 8 μL of the dC chain addition buffer described under 2) was added. After heating at 37°C for 2 min, 1 μL of a terminal transferase (27 units/μL) was added, followed by reaction at 37°C for 3 min. The reaction solution was quickly cooled to 0°C and then 1 μL of 0.5 M EDTA was added to stop the reaction. After phenol-chloroform treatment and ethanol precipitation, the resultant pellet was dissolved in 10 μL of TE buffer.

5) Construction of pBR-based cDNA library

4 μL of a commercially available oligo(dG) chain-added pBR322 vector (Bethesda Research Laboratories, 10 ng/μL) was annealed with 2 μL of the above dC chain-added double-stranded cDNA in 75 μL of 0.1 M NaCl-containing TE buffer. Annealing was performed at 65°C for 5 min and then at 40°C for 2 hr, followed by standing until reaching room temperature.

On the other hand, the method described in Maniatis's book on experimental techniques [Molecular Cloning, Cold Spring Harbor, p. 249 (1982)] was used to prepare competent cells from E. coli strain X1776, to that the above annealed plasmid was transformed, thereby obtaining a transformant.

Practical Example 7: Synthesis of cDNA (construction of λ phage-based cDNA library)

1) Synthesis of single-stranded cDNA

By the method described in Practical Example 5, from 3.8 g of freeze-stored CHU-2 cells, 400 μg of poly(A^+)-RNA was obtained after two rounds of purification on oligo(dT)-cellulose column.

10 μL of TE buffer, dissolving 12 μg of this poly(A^+)-RNA, was placed in a reaction tube containing 10 μg of actinomycin D (Sigma), and then reagents were added in the following order: 20 μL of reverse transcription buffer (250 mM Tris-hydrochloric acid, pH 8.3), 40 mM $MgCl_2$, 250 mM KCl), 20 μL 5 mM dNTP (dATP, dGTP, dCTP and dTTP each at 5 mM), 20 μL of oligo$(dT)_{12\text{-}18}$ (0.2 μg/mL, P-L Biochemicals), 1 μL of 1 M dithiothreitol, 2 μL of 30 units/μL RNase (Promega Biotech), 10 μL of reverse transcriptase (10 units/μL, Seikagaku Kogyo), 1 μL of α-^{32}P-dATP (10 μCi, Amersham), and 16 μL of water (a total of 100 μL of the reaction mixture). After heating at 42°C for 2 hr, 5 μL of 0.5 M EDTA and 1 μL of 20% SDS were added to stop the reaction. After phenol-chloroform treatment (100 μL) and ethanol precipitation (twice), about 4 μg of a single-stranded cDNA was obtained.

2) Synthesis of double-stranded cDNA

The cDNA obtained above was dissolved in 29 μL of TE buffer, and then reagents were added in the following order: 25 μL of polymerase buffer (400 mM Hepes (pH 7.6), 16 mM $MgCl_2$, 63 mM β-mercaptoethanol, 270 mM KCl), 10 μL of 5 mM dNTP, 1.0 μL of 15 mM β-NAD, 1.0 μL of α-^{32}P-dATP (10 μCi/μL), 0.2 μL of E. coli DNA

ligase (60 units/μL, Takara Syuzo), 5.0 μL of E. coli DNA polymerase I (New England Biolabs, 10 units/μL), 0.1 μL of E. coli RNase H (60 units/μL, Takara Syuzo) and 28.7 μL of water.

After incubation at 14°C for 1 hr, the temperature was returned to room temperature, and the mixture was further incubated for 1 hr. Subsequently, 5 μL of 0.5 M EDTA and 1 μL of 20% SDS were added to stop the reaction. After phenol-chloroform treatment and ethanol precipitation, the resultant DNA was dissolved in 20 μL of 0.5 mM EDTA. 3 μL of Klenow buffer (500 mM Tris-hydrochloric acid (pH 8.0), 50 mM $MgCl_2$), 3 μL of 5 mM dNTP and 4 μL of water were added. To this reaction mixture, 1 μL of DNA polymerase (Klenow fragment) (Takara Syuzo) was added, followed by incubation at 30°C for 15 min.

The reaction mixture was diluted with 70 μL of TE buffer. Furthermore, 5 μL of 0.5 M EDTA and 1 μL of 20% SDS were added to stop the reaction. After phenol-chloroform treatment and ethanol precipitation, about 8 μg of double-stranded cDNA was obtained.

3) Methylation of double-stranded cDNA

30 μL of aqueous solution of the double-stranded cDNA synthesized in 2), 40 μL of methylation buffer (500 mM Tris-hydrochloric acid (pH 8.0), 50 mM EDTA), 20 μL of SAM solution (800 μM S-adenosyl-L-methylmethionine (SAM), 50 mM β-mercaptoethanol) and 100 μL of water were mixed. 15 μL of EcoRI methylase (New England Biolabs, 20 units/μL) was added, making the total volume 200 μL, followed by incubation at 37°C for 2 hr. After phenol treatment and ether treatment, ethanol precipitation was performed to recover the DNA.

4) Addition of EcoRI linker

To about 1.2 μg of the double-stranded DNA methylated above, 1.5 μL of ligase buffer (250 mM Tris-hydrochloric acid (pH 7.5), 100 mM $MgCl_2$), 0.5 μL of phosphate-oxidized [sic] EcoRI linker (10 mer, Takara Syuzo), 1.5 μL of 10 mM ATP, 1.5 μL of 100 mM dithiothreitol, and 2 μL of water were added. To the reaction mixture in a volume of 15 μL, 0.7 μL of T4 DNA ligase (3.4 units/μL, Takara Syuzo) was added, followed by reaction at 4°C overnight. The ligase was inactivated by heating at 65°C for 10 min. This reaction mixture was adjusted to a total volume of 50 μL with 100 mM Tris-hydrochloric acid (pH 7.5), 5 mM $MgCl_2$, 50 mM NaCl, 100 μg/mL gelatin, and

then 3.5 μL of EcoRI (10 units/μL) was added. After reaction at 37°C for 2 hr, 2.5 μL of 0.5 M EDTA and 0.5 μL of 20% SDS were added. After phenol-chloroform treatment, ethanol precipitation was performed to recover the DNA. Subsequently, by gel filtration on Ultrogel ACA34 (LKB) or agarose gel electrophoresis, the unreacted EcoRI linker was removed, thereby obtaining 0.5-0.7 μg of a linker-added double-stranded cDNA.

5) Ligation between double-stranded cDNA and λgt10 vector

The above linker-added double-stranded cDNA was mixed with 2.4 μg of EcoRI-treated λgt10 vector (Vector Cloning System), 1.4 μL of ligase buffer (250 mM Tris-hydrochloric acid, 100 mM $MgCl_2$), and 6.5 μL of distilled water. After treatment at 42°C for 15 min, 1 μL of 10 mM ATP, 1 μL of 0.1 M dithiothreitol, and 0.5 μL of T_4 DNA ligase were added, making the total volume 15 μL, followed by reaction overnight at 12°C.

6) In vitro packaging

About 1/3 of the recombinant DNA obtained in above 5) was packaged using In vitro packaging kit (Promega Biotech), thereby obtaining a phage plaque.

Practical Example 8: Screening of pBR-based library with probe (IWQ)

A Whatman No. 541 filter paper was placed on an agar medium with colonies grown. After 2 hr at 37°C, the filter paper was treated by Taub and Thompson's method [Anal. Biochem. Vol. 126, p. 222 (1982).

Thus, after the colonies were transferred to the No. 541 filter paper, they were incubated in an agar medium containing chloramphenicol (250 μg/μL) at 37°C overnight. The No. 541 filter paper was removed, and placed on a filter paper that had been soaked in 0.5 N NaOH solution at room temperature for 3 min. The process was repeated twice. Then, the same procedures were performed twice with 0.5 M Tris-hydrochloric acid (pH 8) solution for 3 min. Furthermore, at 4°C treatment was performed with 0.05 M Tris-hydrochloric acid (pH 8) solution for 3 min, with 1.5 mg/mL lysozyme solution (0.05 M Tris-hydrochloric acid (pH 8), containing 25% sucrose) for 10 min, and then at 37°C with 1 x SSC (0.15 M NaCl and 0.015 M sodium citrate) solution for 2 min, 1 x SSC solution containing 200 μg/mL protease K for 30 min, followed by again at room temperature with 1 x SSC for 2 min and with 95% ethanol solution for 2 min twice. The No. 541 filter paper was dried.

The resultant No. 541 filter paper was soaked in a phenol : chloroform : isoamyl alcohol (25 : 24 : 1) mixture, equilibrated with 100 mM Tris-hydrochloric acid (pH 8.5), 100 mM NaCl, 10 mM EDTA) solution for 30 min. Then, the same procedures were performed with 5 x SSC for 3 min three times, and then with 95% ethanol solution for 3 min twice, followed by drying of the filter paper.

Probe (IWQ) was radiolabeled with ^{32}P by a standard method (see Molecular Cloning), and then colony hybridization was performed by Wallace et al.'s method [Nucleic Acids Res. Vol. 9, p. 879 (1981)]. After pre-hybridization in a hybridization solution containing 6 x NET [0.9 M NaCl, 0.09 M Tris-hydrochloric acid (pH 7.5), 6 mM EDTA], 5 x Denhardt solution, 0.1% SDS, 0.1 mg/mL denatured DNA (fetal bovine thymus DNA) at 65°C for 4 hr, the above hybridization buffer containing the radiolabeled probe (IWQ) at 1 x 10^6 cpm/mL was used to perform hybridization at 56°C overnight. After reaction, the No. 541 filter paper was washed at room temperature with 6 x SSC solution containing 0.1% SDS for 30 min, twice, and then at 56°C for 1.5 min, followed by autoradiography.

From clones with a signal, plasmids were isolated. Probe (IWQ) was used to perform Southern blotting. Hybridization and autoradiography were performed under the same conditions as above.

Similarly, probe (A) was used to perform Southern blotting. Hybridization was performed using the above hybridization buffer at 49°C for 1 hr, and then after cooling to 39°C, at 39°C for 1 hr. After the reaction, the nitrocellulose filter paper was washed at room temperature with 6 x SSC solution containing 0.1% SDS for 30 min, twice and then at 39°C for 3 min, followed by autoradiography.

The results revealed that one clone was positive. The nucleotide sequence was determined by the dideoxy method to be as shown in Fig. 2. It was a DNA comprising 308 base pairs, containing both probe (IWQ) and probe (A) sequences. The pBR322-derived plasmid containing this insert was named as PHCS-1.

Practical Example 9: Screening of λ-phage-based cDNA library using PHCS-1-derived DNA probe

Plaque hybridization was performed in accordance with Benton and Davis's method [Science, Vol 196, p. 180 (1977)]. PHCS-1 obtained in Practical Example 8 was

treated with Sau3A and EcoRI to obtain an about 600 base pair DNA fragment. This DNA fragment was radiolabeled by nick translation by a standard method. A nitrocellulose filter paper (S & S) was placed on an agar medium with phage plaques grown to transfer the phages. After denaturing DNA with 0.5 M NaCl, the filter paper was treated in the following order: 0.1 M NaOH and 1.5 M NaCl for 20 sec, 0.5 M Tris-hydrochloric acid (pH 7.5), 1.5 M NaCl for 20 sec twice, and finally 120 mM NaCl, 15 mM sodium citrate, 13 mM KH_2PO_4 and 1 mM EDTA (pH 7.2) for 20 sec.

Subsequently, the filter paper was dried, and heated at 80°C for 2 hr to immobilize the DNA. Pre-hybridization was performed in a hybridization solution containing 5 x SSC, 5 x Denhardt solution, 50 mM phosphate buffer, 50% formamide, 0.25 mg/mL denatured DNA (salmon sperm DNA) and 0.1% SDS at 42°C overnight. Hybridization was performed in a hybridization buffer (5 x SSC, 5 x Denhardt solution, 20 mM phosphate buffer (pH 6.0), 50% formamide, 0.1% SDS, 10% dextran sulfate and 0.1 mg/mL denatured DNA (salmon sperm DNA) containing 4 x 10^5 cpm/mL of the nick translation radiolabeled pHCS-1 probe, at 42°C for 20 hr.

The nitrocellulose filter paper was washed at room temperature with 2 x SSC solution containing 0.1% SDS for 20 min, and then at 44°C with 0.1 x SSC containing 0.1% SDS for 30 min and furthermore at room temperature with 0.1 x SSC for 10 min, followed by detection using autoradiography.

The results revealed that five clones were positive (G1-5). The DNA sequence of a clone considered to contain a full-length cDNA was determined, thereby obtaining the nucleotide sequence shown in Fig. 3(A). This cDNA was cut out of the λgt10 vector, and ligated with pBR327 [Soberon et al.: Gene, Vol. 9, p. 287 (1980)] at the EcoRI site. The plasmid was prepared in a large quantity, and this plasmid was named pBRG4.

Practical Example 10: Screening λ phage-based cDNA library using pBRG4-derived DNA probe and probe (LC)

Plaque hybridization was performed in accordance with Benton and Davis's method used in Practical Example 9 (see above mentioned reference). A nitrocellulose filter paper (S & S) was placed on an agar medium with phage plaques grown to transfer the phages. After denaturing DNA with 0.5 M NaOH, the filter paper was treated in the following order: 0.1 M NaOH and 1.5 M NaCl for 20 sec, 0.5 M Tris-hydrochloric acid (pH 7.5), 1.5 M NaCl for 20 sec twice, and finally 120 mM NaCl, 15 mM sodium citrate, 13 mM

KH_2PO_4 and 1 mM EDTA (pH 7.2) for 20 sec. Subsequently, the filter paper was dried, and heated at 80°C for 2 hr to immobilize the DNA. Two identical filter papers were prepared in this way, for screening with pBRG4-derived DNA probe and probe (LC), respectively. When the pBRG4-derived DNA probe was used, pBRG4 was treated with EcoRI to obtain a DNA fragment with about 1500 base pairs. This DNA fragment was radiolabeled by nick translation using a standard method. The above filter paper was subjected to pre-hybridization in a hybridization solution containing 5 x SSC, 5 x Denhardt solution, 50 mM phosphate buffer, 50% formamide, 0.25 mg/mL denatured DNA (salmon sperm DNA) and 0.1% SDS at 42°C overnight. Hybridization was performed in hybridization buffer [(5 x SSC, 5 x Denhardt solution, 20 mM phosphate buffer (pH 6.0), 50% formamide, 0.1% SDS, 10% dextran sulfate and 0.1 mg/mL denatured DNA (salmon sperm DNA] containing the above radiolabeled about 1500 base pairs DNA probe (about 1 x 10^6 cpm/mL) at 42°C for 20 hr. The nitrocellulose filter paper was washed at room temperature with 2 x SSC solution containing 0.1% SDS for 20 min, and then at 44°C with 0.1 x SSC containing 0.1% SDS for 30 min and furthermore at room temperature with 0.1 x SSC for 10 min, followed by detection by autoradiography.

When probe (LC) was used, the filter paper was subjected to pre-treatment in 3 x SSC containing 0.1% SDS for 2 hr, and then to pre-hybridization in a buffer containing 6 x NET, 1 x Denhardt solution, 100 μg/mL denatured DNA (salmon sperm DNA) at 65°C for 2 hr.

Hybridization was performed in hybridization buffer [6 x NET, 1 x Denhardt solution, 100 μg/mL denatured DNA (salmon sperm DNA] containing the above radiolabeled probe (LC) (2 x 10^6 cpm/mL) at 63°C overnight. The nitrocellulose filter paper was washed at room temperature with 6 x SSC solution containing 0.1% SDS for 20 min. After washing 3 times with this solution, 6 x SSC containing 0.1% SDS was used for washing at 63°C for 2 min.

The filter was dried and then subjected to autoradiography for detection.

By the screening performed above, clones positive with both probes were selected. The DNA sequence of a clone considered to contain a full-length cDNA was determined using the dideoxy method, thereby obtaining the nucleotide sequence shown

in Fig. 4(A). This cDNA was cut out of the λgt10 vector, and ligated with pBR327 at the EcoRI site, thereby obtaining a plasmid named pBRV2.

Practical Example 11: Screening of human chromosomal gene library

1) Construction of human chromosomal gene library

A human chromosomal gene library was provided by Maniatis (Harvard University), which was prepared as follows.

Total chromosomal DNA was extracted with phenol, etc. from human fetal liver, and then digested with the restriction enzymes HaeIII and AluI. From the DNA fragments thus obtained, fragments with a length of about 18-25 kb were enriched by sucrose density gradient centrifugation. Subsequently, it was ligated to the arm DNA of the E. coli phase λ Charon 4A via a short chain synthetic nucleotide containing the cutting site of the restriction enzyme EcoRI, thereby preparing an infectious recombinant phage DNA. Then, to further enhance the infecting capability, it was converted to a complete phage λ plasmid by the packaging method. The human gene library thus prepared can be considered to be a recombinant human DNA collection with a length of 18-25 kb including in principle almost all human genes.

2) Screening of human chromosomal gene library with pHCS-1-derived DNA probe

Plaque hybridization was performed by Benton and Davis' method [Science, Vol. 196, p. 180 (1977)]. PHCS-1 obtained in Practical Example 8 was treated with Sau3A and EcoRI to obtain an about 600 bp DNA fragment. This DNA fragment was radiolabeled by nick translation. A nitrocellulose filter paper (S & S) was placed on agar medium with phage plaque grown to transfer the phage. DNA was denatured with 0.5 M NaOH and then the filter paper was treated with the following, in this order: 0.1 M NaOH, 1.5 M NaCl for 20 sec, 0.5 M Tris hydrochloric acid (pH 7.5), 1.5 M NaCl for 20 sec twice, and finally 120 mM NaCl, 15 mM sodium citrate, 13 mM KH_2PO_4, 1 mM EDTA (pH 7.2) for 20 sec.

Subsequently, the filter paper was dried, and heated at 80°C for 2 hr to immobilize DNA. Hybridization was performed at 42°C overnight in a hybridization solution containing 5 x SSC, 5 x Denhardt solution, 50 mM phosphate buffer, 50% formamide, 0.25 mg/mL denatured DNA (salmon sperm DNA) and 0.1% SDS, followed by hybridization with pHCS-1 probe radiolabeled by nick translation at 4 x 10^5 cpm/mL in hybridization buffer [(5 x SSC, 5 x Denhardt solution, 20 mM phosphate buffer (pH

6.0), 50% formamide, 0.1% SDS, 10% dextran sulfate and 0.1 mg/mL denatured DNA (salmon sperm DNA] at 42°C for 20 hr.

The nitrocellulose filter paper was washed at room temperature with 2 x SSC solution containing 0.1% SDS for 20 min, and then at 44°C with 0.1 x SSC containing 0.1% SDS for 30 min and furthermore at room temperature with 0.1 x SSC for 10 min, followed by detection by autoradiography.

A dozen or so positive clones were obtained by these procedures.

From these clones, the recombinant DNA was prepared by Maniatis' method [Cell, Vol. 15, p. 687 (1978)].

The resultant DNA was treated with the restriction enzymes EcoRI, BamHI, BglII, etc. By analysis on agarose gel electrophoresis, a restriction enzyme map was prepared by Fritsch et al.'s method (see the above-mentioned reference).

The radiolabeled pHCS-1-derived DNA fragment probe used in the above screening was used to perform Southern blotting. An about 8 kb DNA fragment cut by EcoRI was selected from those hybridizing with the probe, and was subcloned into pBR327 at the EcoRI site.

Furthermore, the subcloned DNA was treated with restriction enzymes, followed by Southern blotting. Repeating these procedures revealed that the gene encoding human G-CSF polypeptide existed in an about 4 kb DNA fragment cut out by EcoRI and XhoI.

Using the dideoxy method the sequence of about 3 kb of this DNA fragment was determined, thereby obtaining the nucleotide sequence shown in Fig. 5.

The restriction enzyme cutting sites of this DNA fragment are shown in Fig. 7.

Besides the above probe, pBRG4-derived DNA and pBRV2-derived DNA were also used for the screening of human chromosomal genes.

Both DNAs, 1500 bp DNA fragments after treated with EcoRI, were radiolabeled by nick translation. Or, an about 700 bp DNA fragment obtained after EcoRI treatment

and subsequent DraI treatment was similarly radiolabeled. Under the same conditions as above, plaque hybridization was performed to select clones. Using Southern blot analysis, a DNA fragment with the nucleotide sequence shown in Fig. 5 was obtained. The resultant plasmid was named pBRCE3β.

Practical Example 12: Preparation of pHGA410 vector (for animal cells, +VSE system)

The EcoRI fragment of the cDNA obtained in Practical Example 9 and shown in Fig. 3 (A) was digested with the restriction enzyme DraI at 37°C for 2 hr, and then treated with the Klenow fragment of DNA polymerase I (Takara Syuzo) to convert the terminus into a blunt end. 1 μg of BglII linker (8 mer, Takara Syuzo) was phosphorylated with ATP, and then ligated with the about 1 μg of the DNA fragment mixture obtained above. Subsequently, after treatment with the restriction enzyme BglII, agarose gel electrophoresis was performed to recover the largest DNA fragment.

This DNA fragment was about 710 base pairs containing the region encoding human G-CSF polypeptide as shown in Fig. 6. The vector pdKCR [Fukunaga et al.: Proc. Natl. Acad. Sci. USA, Vol. 81, p. 5086 (1984)] was treated with the restriction enzyme BamHI and then dephosphorylated with alkaline phosphatase (Takara Syuzo). To the resultant vector DNA, T4 DNA ligase (Takara Syuzo) was added to ligase the cDNA fragments, thereby obtaining pHGA410 (Fig. 8). As shown in Fig. 8, this plasmid contained the initial gene promoter of SV40, replication origin of SV40, a part of rabbit β-globulin gene, replication origin of pBR322 and pBR322-derived β-lactamase gene (Amp^r), with the human G-CSF gene located downstream of the initial gene promoter of SV40.

Practical Example 13: Construction of recombinant vector for C127 cells (+VSE)

1) Construction of pHGA410 (H)

20 μg of the pHGA410 plasmid obtained in Practical Example 12 (Fig. 8) was dissolved in a reaction solution (50 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 100 mM NaCl, 7 mM 2-mercaptoethanol, 0.01% bovine serum albumin (BSA)), and the restriction enzyme EcoRI (Takara Syuzo, 10-15 units) was added. After reaction at 37°C for about 30 min to perform partial digestion with EcoRI, phenol-chloroform (1:1) treatment was performed twice, followed by ether treatment. After ethanol precipitation, a DNA fragment was obtained.

This DNA fragment was dissolved in 50 μL of a solution consisting of 50 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM DTT, 1 mM dATP, dCTP, dGTP and dTTP, and 5 μL of Klenow fragment of E. coli DNA polymerase (Takara Syuzo) was added. By incubation at 14°C for 2 hr, the terminus was converted to a blunt end.

By 0.8% agarose gel electrophoresis, 6 μg of an about 5.8 kb fragment was recovered.

5 μg of the recovered DNA fragment was dissolved in 50 μL of a reaction solution consisting of 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, and 2 μg of HindIII linker (Takara Syuzo) and 100 units of T_4 DNA ligase (Takara Syuzo) were added. After reaction at 4°C overnight, phenol treatment, ether treatment and then ethanol precipitation were performed, followed by dissolution in 30 μL of a solution consisting of 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 60 mM NaCl. 10 units of the restriction enzyme HindIII was added and the mixture was incubated at 37°C for 3 hr. After another T4 DNA ligase treatment, this DNA was transformed in E. coli strain DH1 using the rubidium chloride method (see above-mentioned Molecular Cloning). Ampicillin-resistant (Amp^r) colonies were obtained and bacterial cells retaining the plasmid with the EcoRI site of pHGA410 plasmid replaced by HindIII site were selected. The plasmid thus obtained was named pHGA410 (H) (Fig. 9).

2) Construction of expression vector pTN-G4

PHGA410 (H) obtained in above 1) (20 μg) was dissolved in 50 μL of a reaction solution consisting of 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 175 mM NaCl, 0.2 mM EDTA, 7 mM 2-mercaptoethanol, 0.01% bovine serum albumin, and 20 units of the restriction enzyme SalI (Takara Syuzo) was added. After incubation at 37°C for 5 hr, phenol treatment and ethanol precipitation were performed, followed by incubation with Klenow fragment of E. coli DNA polymerase (Takara Syuzo), as with the above reaction, at 14°C for 2 hr to prepare a blunt end. Without recovery by agarose gel electrophoresis, the DNA fragment precipitated with ethanol was treated with the restriction enzyme HindIII. Thus, 5 μg of an about 2.7 kb HindIII-SalI fragment was recovered by 1% agarose gel electrophoresis.

On the other hand, the plasmid pdBPV-1 containing bovine papilloma virus (BPV) [Sarver, M., Sbyrne, J. C. & Howley, P. M. (1982) Proc. Natl. Acad. Sci. USA, Vol. 79, p. 7147-7151; from Dr. Howley] was treated with HindIII and PvuII by Nagata

et al.'s method [Fukunaga, Sokawa & Nagata (1984) Proc. Natl. Acad. Sci. USA, Vol. 81, p. 5086-5090], thereby obtaining a 8.4 kb DNA fragment.

This 8.4 kb DNA fragment and the above about 2.7 kb HindIII-SalI DNA fragment were treated with T4 DNA ligase by a standard method. After transformation in E. coli strain DH1 using the rubidium chloride method (see above mentioned Molecular Cloning), E.coli colonies retaining the pHGA410-derived G-CSF cDNA were selected. This plasmid was named pTN-G4 (Fig. 9).

On the other hand, from the plasmid ΔpVA containing an about 1700 bp SalI-HindIII fragment containing VAI and VAII of adenovirus type II [Protein, Nucleic Acid and Enzyme, Vol. 27, No. 12 (1982)], the fragment containing VAI and VAII was recovered. This fragment was inserted into the HindIII site of the above pTNG4, thereby obtaining pTNG4VAα and pTNG4VAβ (Fig. 9). This plasmid has enhanced expression of the gene product driven by the initial promoter of SV40 due to the VA gene of adenovirus.

Practical Example 14: Transfection of and expression in C127 cells (+VSE)

pTN-G4 obtained in Practical Example 13 was treated with the restriction enzyme BamHI in advance before transfection in mouse C127 cells. Thus, 20 μg of pTN-G4 plasmid was dissolved in 100 μL of a solution consisting of 10 mM Tris-HCl (pH 8.0), 7 mM $MgCl_2$, 100 mM NaCl, 2 mM 2-mercaptoethanol, 0.01% BSA, and then treated with 20 units of BamHI (Takara Syuzo), followed by phenol treatment, ether treatment and then ethanol precipitation.

Mouse C127I cells were grown in Dulbecco's minimal essential medium containing 10% bovine fetal serum (GIBCO). In C127I cells grown in a plate with a diameter of 5 cm, 10 μg per plate of the DNA prepared above was transfected using the phosphoric acid-calcium method [Haynes, J. & Weissmann, C. (1983) Nucleic Acid Res. Vol. 11, p. 687-706]. After glycerol treatment, incubation was performed at 37°C for 12 hr.

Subsequently, the cells were transferred to three fresh plates with a diameter of 5 cm, and cultured with the medium being changed twice a week. On day 16 those with foci formed were transferred to fresh plates, and cultured through passages with the above medium. Clones with high production of G-CSF were selected. The results

showed production of G-CSF at a level of about 1 mg/l. Further cloning was continued, and production of G-CSF at a level of at least 10 mg/l was confirmed.

Besides the above C127I cells, NIH3T3 can also be used as host cell.

Practical Example 15: Expression of G-CSF in CHO cells (+VSE)

1) Construction of pHGG4-dhfr

20 μg of the pHGA410 plasmid obtained in Practical Example 12 was dissolved in 100 μL of a reaction solution (10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 175 mM NaCl, 0.2 mM EDTA, 0.7 mM 2-mercaptoethanol, 0.01% BSA), and 20 units of the restriction enzyme SalI (Takara Syuzo) were added. After reaction at 37°C overnight, phenol treatment, ether washing and then ethanol precipitation were performed.

Subsequently, the resultant DNA precipitate was dissolved in 100 μL of a reaction solution consisting of 50 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM DTT, 1 mM dATP, dCTP, dGTP and TTP, and the Klenow fragment of E. coli DNA polymerase (Takara Syuzo, 10 μL) was added. After reaction at 14°C for 2 hr, phenol treatment, ether washing and then ethanol precipitation were performed.

To the DNA, an EcoRI linker was added. Thus, the above DNA was dissolved in 50 μL of a reaction solution consisting of 50 mM Tris-HCl (pH 7.4), 10 mM DTT, 0.5 mM spermidine, 2 mM ATP, 2.5 mM cobalt hexammine chloride, 20 μg/mL BSA, and an EcoRI linker (Takara Syuzo) was added. 200 units of T4 DNA ligase (Takara Syuzo) were added, and the reaction was performed at 4°C for 12-16 hr. After phenol treatment, ether washing and then ethanol precipitation by a standard methods, the DNA was partially digested with EcoRI. Using 1% agarose gel electrophoresis 3 μg of an about 2.7 kb fragment was recovered.

On the other hand, pAdD26SVpA plasmid [Kaufman, R. G. & Sharp, P. A. (1982) Mol. Cell. Biol. Vol. 2, p. 1304-1319] was treated with EcoRI, and then with bacterial alkaline phosphatase (BAP) for dephosphorylation. Thus, 20 μg of pAdD26SVpA and 20 units of EcoRI were added to 100 μL of a reaction solution consisting of 50 mM Tris-HCl (pH 7.5), 7 mM MgCl2, 100 mM NaCl, 7 mM 2-mercaptoethanol, 0.01% BSA, and the reaction was performed at 37°C for 10 hr. 5 units of BAP were added to the reaction solution, followed by incubation at 68°C for 30 min.

Subsequently, after phenol treatment, by electrophoresis the EcoRI fragment of pAdD26SVpA was recovered (about 5 μg).

The above about 2.7 kb fragment and the pAdD26SVpA fragment, each 0.5 μg, were annealed. This plasmid was transformed in E. coli strain DH1 using the rubidium chloride method. Colonies retaining the pHGG4-dhfr plasmid were selected. The plasmid thus obtained was named pHGG4-dhfr (Fig. 10a).

Separately, the pHGG4 plasmid was treated with SalI. Without the addition of EcoRI linker, partial digestion with EcoRI was performed to recover an about 2.7 kb fragment. The DNA fragment was treated with the Klenow fragment of E. coli DNA polymerase to obtain a blunt end.

On the other hand, by the above method an EcoRI fragment with a blunt end was prepared from pAdD26SVpA. By treating the two fragments with T4 DNA ligase, pHGG4-dhfr can also be prepared

Alternatively, the pHGA410 (H) obtained in 1) of Practical Example 13 was treated with the restriction enzymes HindIII and SalI as described in 2) of Practical Example 13. By ligating the HindIII-SalI fragment with the EcoRI fragment with a blunt end from pAdD26SVpA, pHGG4-dhfr can also be prepared (Fig. 10b).

2) Construction of pG4DR1 and pG4DR2

10 μg of pAdD26SVpA described in 1) was dissolved in 50 μL of a reaction solution consisting of 50 mM Tris-HC1 (pH 7.5), 7 mM $MgCl_2$, 100 mM NaCl, 7 mM 2-mercaptoethanol, 0.01% BSA), and 10 units each of the restriction enzymes EcoRI and BamHI were added. After reaction at 37°C for 10 hr, phenol treatment and ether wash were performed by standard methods. By 1% low melting point agarose gel electrophoresis, an about 2 kb DNA fragment was recovered. A blunt end was prepared using the Klenow fragment of DNA polymerase by a standard method, followed by phenol treatment, ether washing and then ethanol precipitation.

On the other hand, 10 μg of pHGA410 (H) obtained in 1) of Practical Example 13 was dissolved in 50 μL of a reaction solution consisting of 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 60 mM NaCl, and 10 units of HindIII were added. After reaction at 37°C for 6 hr, by 1% low melting point agarose gel electrophoresis by a standard method, the

DNA fragment was recovered. After further BAP treatment, a blunt end was prepared using Klenow fragment. After phenol treatment and ether washing, it was ligated with the above described about 2 kb DNA fragment using T_4 DNA ligase by blunt end ligation. Thus, 1 μg each of the DNA fragments were dissolved in 30 μL of a reaction solution consisting of 66 mM Tris-HCl (pH 7.5), 6.6 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, and 50 units of T4 DNA ligase were added. After reaction at 6°C for 12 hr, transformation of E. coli strain DH1 was performed. Thus, pG4DR1 and pG4DR2 shown in Fig. 10c were obtained.

3) Transfection and expression

CHO cells (dhfr$^-$ strain, from Dr. L. Chasin of Columbia University) were grown in α minimal essential medium (α-MEM, with addition of adenosine, deoxyadenosine, thymidine) containing 10% bovine fetal serum in a plate with a diameter of 9 cm (Nunc). The cells were transfected by the phosphoric acid-calcium method [Wigler et al.: Cell, Vol. 14, p. 725 (1978)].

Thus, to 1 μg of pHGG4-dhfr prepared in 1), an appropriate amount of a carrier DNA (fetal bovine thymus DNA) was added, followed by dissolution in 375 μL of TE buffer. 125 μL of 1 M $CaCl_2$ was added. After cooling on ice for 3-5 min, 500 μL of 2 x HBS (50 mM Hepes, 280 mM NaCl, 1.5 mM phosphate buffer) was added. The mixture was cooled again, and then mixed with 1 mL of the above CHO culture. After placing in a plate, culture was performed in a CO_2 incubator for 9 hr. The medium was removed from the plate. After washing with TBS (Tris-buffered saline), TBS containing 20% glycerol was added. After washing again, a non-selective medium (the above α-MEM medium, with nucleotide addition) was added. After incubation for 2 days, the cells were divided at 1:10 with a selective medium (without nucleotide addition). Then, the medium was replaced with the selective medium once every 2 days to continue the culture. Newly formed colonies were selected and transferred to a fresh plate.

In the fresh plate, cell grow was performed in the presence of 0.02 μM methotrexate (MTX). Cells were then grown in the presence of 0.05 μM and then even of 0.1 μM MTX to perform cloning. The transfection of CHO cells can also be performed by co-transfection of pHGG4 and pAdD26SVpA in CHO cells [Scahill et al.: Proc. Natl. Acad. Sci. USA, Vol. 80, p. 4654-4658 (1983)].

Alternatively, by the following method, the transfection of CHO cells can also be performed. Thus, pG4DR1 or pG4DR2 prepared in above 2) was treated with the restriction enzymes SalI and KpnI in advance to obtain a DNA fragment. 10 μg of the fragment was transfected in CHO cells as above. Cells thus transfected were cultured as above in selective medium. On day 7 at least 100 colonies per plate clearly appeared. Without selecting colonies one by one, they were transferred to a fresh plate, and cultured in the presence of 0.01 μM MTX. A dozen or so colonies appeared. In this way, the concentration of MTX was increased to 0.02 μM, 0.05 μM and 0.1 μM to select surviving colonies. Alternatively, the colonies can also be selected by increasing MTX concentration after selecting the resultant a dozen or so colonies.

Moreover, a recombinant vector with the so-called polycistronic gene was prepared, and transfected in CHO cells. Thus, pAdD26SVpA was treated with PstI to recover two fragments, that were ligated with the CSF cDNA fragment derived from pBRG4, thereby constructing a recombinant vector with adenovirus promoter, CSF cDNA, DHFR and polyA site of SV40 (in this order). The vector was transfected into CHO cells.

Practical Example 16: Identification of activity of expressed G-CSF (+VSE)

Culture supernatants of the C127 and CHO cells obtained in Practical Examples 14 and 15, respectively, were adjusted to pH 4 with 1 N acetic acid. An equal volume of n-propanol was added. The resultant precipitate was removed by centrifugation, and the sample was applied to an open column (1 ϕ x 2 cm) packed with a C8 reverse phase carrier (Yamamura Chemicals). Elution was performed with 50% n-propanol. The eluate was diluted 2-fold with water, and then applied to reverse phase high performance liquid chromatography using a YMC-C8 column (Yamamura Chemicals). Elution was performed with a linear concentration gradient of n-propanol at 30-60% in 0.1% TFA. Fractions at an n-propanol concentration of about 40% were collected and freeze-dried, followed by dissolution in 0.1 M glycine buffer (pH 9). By these procedures, human G-CSF was enriched about 20-fold from the supernatants of C127 and CHO cells.

For control, human G-CSF cDNA-free plasmid was transfected by the above method and the culture supernatant was concentrated. The resultant samples were subjected to human G-CSF assay by the method described in the Reference Example "Assay for human G-CSA (a)". For very high level expression, the culture supernatant

can be used directly for the assay. Here, results of examples using concentrated samples are shown, in Table 1.

Table 1: Testing of human G-CSF activity

		Human neutrophil colony number/dish
Purified human G-CSF (20 ng)		96
BPV	Culture supernatant of pdBPV-1-transfected C127 cells (concentrated 20-fold)	0
	Culture supernatant of pdBPV-1-transfected 3T3 cells (concentrated 20-fold)	0
	Culture supernatant of pTNG4-transfected C127 cells (concentrated 20-fold)	82
	Culture supernatant of pTNG4-transfected 3T3 cells (concentrated 20-fold)	85
dhfr	Culture supernatant of pAdD26SVpA-transfected CHO cells (concentrated 20-fold)	0
	Culture supernatant of pHGG4-dhfr-transfected CHO cells (concentrated 20-fold)	110
	Culture supernatant of pG4DR1-transfected CHO cells (concentrated 20-fold)	105

Practical Example 17: Amino acid analysis and carbohydrate analysis (+VSE)

1) Analysis of amino acid composition

The crude CSF sample obtained in Practical Example 16 was further purified by the method of (iii) of Practical Example 2. The purified CSF sample was hydrolyzed by a standard method. The amino acid composition of the protein was analyzed using a Hitachi Model 835 Amino Acid Automated Analyzer (Hitachi Production) by the special amino acid analysis method. The results are shown in Table 2. The hydrolysis conditions were as follows.

(1) 6 N HCl, 110°C, 24 hr, in vacuum;

(2) 4 N methanesulfonic acid + 0.2% 3-(2-aminoethyl)indole, 110°C, 24 hr, 48 hr, 72 hr, in vacuum.

The sample was dissolved in 0.1% trifluoroacetic acid containing 40% n-propanol (1.5 mL). 0.1 mL each was dried with dry nitrogen gas, and then reagent (1) or (2) was added. The tube was sealed in vacuum, and then subjected hydrolysis.

In the table, the data are the averages of four measurements: (1) 24 hr; and (2) 24, 48 and 72 hr. However, the data for Thr, Ser, ½ Cys, Met, Val, Ile and Trp were calculated as follows (see Biochemical Experiment Series, Protein Chemistry II (Tokyo Kagaku Dojin)).

- For Thr, Ser, ½ Cys and Met, the time course of (2) 24, 48 and 72 hr values was measured, and then extrapolated to 0 hr.
- For Val and Ile, the (2) 72 hr values were used.
- For Trp, the average of the (2) 24, 48 and 72 hr values was used.

Table 2 (Amino acid analysis table)

Amino acid	mol%
Asp (Asp+Asn)	2.3
Thr	3.9
Ser	8.5
Glu (Glu+Gln)	15.3
Pro	7.4
Gly	7.8
Ala	10.8
1/2 Cys	2.8
Val	4.5
Met	1.7
Ile	2.3
Leu	18.6
Tyr	1.7
Phe	3.4
Lys	2.3
His	2.8
Trp	1.1
Arg	2.8

2) Carbohydrate composition analysis

To 200 ng of the above purified CSF sample used for amino acid analysis, 25 nmol of inositol was added as internal standard. Methanol containing 1.5 N HCl (500 μL) was added, and the tube was sealed after the air was replaced with nitrogen gas. After reaction at 90°C for 4 hr, the tube was opened and silver carbonate (Ag_2CO_3) was added for neutralization. 50 μL of anhydrous acetic acid was added and shaken, followed by standing at room temperature overnight in the dark. The upper phase was removed to a sample tube and dried with nitrogen gas. The residue was washed with methanol and

lightly centrifuged. The upper phase was dried in the same sample tube. 50 μL of TMS reagent (pyridine : hexamethylsilazane : trimethylchlorosilane = 5:1:1) was added. After reaction at 40°C for 20 min, the mixture was stored in a deep freezer. Galactose (Gal), N-acetylgalactosamine (GalNAc), sialic acid, each 50 nmol, and inositol, 25 nmol, were combined and subjected to the same procedures as standards.

The samples were subjected to gas chromatography analysis under the following conditions.

(Analysis conditions)
Column: 2%OV-17 VINport HP60-80 mesh, 3 m, glass
Temperature: an increase of 4°C/min over 110°C – 250°C
Carrier gas: initially 1.2-1.6 kg/cm^2 (nitrogen gas pressure), at the end 2-2.5 kg/cm^2
Sensitivity: 10^3 MQ range 0.1-0.4 V
Pressure: hydrogen gas 0.8 kg/cm^2
air 0.8 kg/cm^2
Sample volume: 2.5-3.0 μL

Analysis results confirmed the presence of galactose, N-acetylgalactosamine and sialic acid in the CSF of the present invention.

Practical Example 18: Preparation of the pHGV2 vector (for animal cells, -VSE system)

The EcoRI fragment of the cDNA obtained in Practical Example 10 and shown in Fig. 4 (A) was digested with the restriction enzyme DraI at 37°C for 2 hr, and then treated with the Klenow fragment of DNA polymerase I (Takara Syuzo) to convert the terminus into a blunt end. Then, 1 μg of BglII linker (8 mer, Takara Syuzo) was phosphorylated with ATP, and then ligated with the about 1 μg of the DNA fragment mixture obtained above. Subsequently, after treatment with the restriction enzyme BglII, agarose gel electrophoresis was performed to recover the largest DNA fragment.

This DNA fragment was about 700 base pairs containing the region encoding human G-CSF polypeptide as shown in Fig. 6. The vector -pdKCR [Fukunaga et al.: Proc. Natl. Acad. Sci. USA, Vol. 81, p. 5086 (1984)] was treated with the restriction enzyme BamHI and then dephosphorylated with alkaline phosphatase (Takara Syuzo). To the resultant vector DNA, T4 DNA ligase (Takara Syuzo) was added to ligate the

cDNA fragments, thereby obtaining pHGV2 (Fig. 11). As shown in Fig. 11, this plasmid contained the initial gene promoter of SV40, replication origin of SV40, a part of rabbit β-globulin gene, replication origin of pBR322 and pBR322-derived β-lactamase gene (Amp^r), with the human G-CSF gene located downstream of the initial gene promoter of SV40.

Practical Example 19: Construction of recombinant vector for C127 cells (-VSE)

1) Construction of pHGV2 (H)

20 μg of the pHGV2 plasmid obtained in Practical Example 18 (Fig. 11) was used to obtain a plasmid named pHGV2 (H) (Fig. 12), using the same method as described in 1) of Practical Example 13.

2) Construction of expression vector pTN-V2 and construction of pTNVAα and pTNVAβ

The pHGV2 (H) obtained in above 1) (20 μg) was used to select E. coli colonies retaining a plasmid containing the pHGV2-derived G-CSF cDNA by the same method as described in 2) of Practical Example 13. The plasmid obtained was named as pTN-V2 (Fig. 12).

On the other hand, from the plasmid ΔpVA containing an about 1700 bp SalI-HindIII fragment containing VAI and VAII of adenovirus type II [Protein, Nucleic Acid and Enzyme, Vol. 27, No. 12 (1982)], the fragment containing VAI and VAII was recovered. This fragment was inserted into the HindIII site of the above pTN-V2, thereby obtaining pTNVAα and pTNVAβ (Fig. 12). This plasmid has enhanced expression of the gene product driven by the initial promoter of SV40 due to the VA gene of adenovirus.

Practical Example 20: Transfection of and expression in C127 cells (-VSE)

The pTNV2 obtained in Practical Example 19 was treated with the restriction enzyme BamHI in advance before transfection in mouse C127 cells.

Then, the DNA prepared above was transfected in mouse C127I cells (see Practical Example 14) to select clones with high G-CSF production. The results showed production of G-CSF at a level of about 1 mg/L.

Further cloning was continued, and a clone producing G-CSF at a level of 10 mg/l was selected. Similarly, pTNVAα and pTNVAβ obtained in Practical Example 19 were

transfected in C127 cells to select clones with high G-CSF production. The results showed that clones were obtained with pTNVAα with production of G-CSF at a level of at least 20 mg/L. With pTNVAβ, clones with production of G-CSF at a level of several mg/L were obtained.

Besides the above C127I cells, NIH3T3 can also be used as host cell.

Practical Example 21: Expression of G-CSF in CHO cells (-VSE)

1) Construction of pHGV2-dhfr

From 20 μg of the pHGV2 plasmid obtained in Practical Example 18, an about 2.7 kb fragment was obtained by the method described in 1) of Practical Example 15, and then this was annealed with the fragment from pAdD26SVpA, each 0.5 μg.

This plasmid was transformed in E. coli strain DHI by the rubidium chloride method. Colonies retaining the pHGV2-dhfr plasmid were selected. The plasmid thus obtained was named pHGV2-dhfr (Fig. 13a).

Separately, the pHV2 plasmid was treated with SalI. Without the addition of EcoRI linker, partial digestion with EcoRI was performed to recover an about 2.7 kb fragment. The DNA fragment was treated with Klenow fragment of E. coli DNA polymerase to obtain a blunt end.

On the other hand, by the above method an EcoRI fragment with a blunt end was prepared from pAdD26SVpA. By treating the two fragments with T4 DNA ligase, pHGV2-dhfr can also be prepared.

Alternatively, pHGV2 (H) obtained in 1) of Practical Example 19 was treated with the restriction enzymes HindIII and SalI as described in 2) of Practical Example 13. By ligating the HindIII-SalI fragment with the EcoRI fragment with a blunt end from pAdD26SVpA, pHGV2-dhfr can also be prepared (Fig. 13b).

2) Construction of pV2DR1 and pV2DR2

10 μg of pAdD26SVpA described in 1) was dissolved in 50 μL of a reaction solution consisting of 50 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 100 mM NaCl, 7 mM 2-mercaptoethanol, 0.01% BSA), and 10 units each of the restriction enzymes EcoRI and BamHI were added. After reaction at 37°C for 10 hr, phenol treatment and ether wash

were performed by standard methods. By 1% low melting point agarose gel electrophoresis an about 2 kb DNA fragment was recovered. A blunt end was prepared using the Klenow fragment of DNA polymerase by a standard method, followed by phenol treatment, ether washing and then ethanol precipitation.

On the other hand, 10 μg of pHGV2 (H) obtained in 1) of Practical Example 19 was dissolved in 50 μL of a reaction solution consisting of 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 60 mM NaCl, and 10 units of HindIII was added. After reaction at 37°C for 6 hr, by 1% low melting point agarose gel electrophoresis using a standard method, the DNA fragment was recovered. After further BAP treatment, a blunt end was prepared using Klenow fragment. After phenol treatment and ether wash, it was ligated with the above-described about 2 kb DNA fragment using T4 DNA ligase by blunt end ligation. Thus, 1 μg each of the DNA fragments were dissolved in 30 μL of a reaction solution consisting of 66 mM Tris-HCl (pH 7.5), 6.6 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, and 50 units of T4 DNA ligase were added. After reaction at 6°C for 12 hr, transformation of E. coli strain DH1 was performed. Thus, pV2DR1 and pV2DR2 shown in Fig. 13c were obtained.

3) Transfection and expression

The pHGV2-dhfr plasmid prepared in above 1) was used to perform transfection in CHO cells by the same method as described in 3) of Practical Example 15.

The transfection of CHO cells can also be performed by co-transfection of pHGV2 and pAdD26SVpA in CHO cells.

Alternatively, by the following method, the transfection of CHO cells can also be performed. Thus, pV2DR1 or pV2DR2 prepared in above 2) was treated with the restriction enzymes SalI and KpnI in advance to obtain a DNA fragment. 10 μg of the fragment was transfected in CHO cells as above. Cells thus transfected were cultured as above in a selective medium. On day 7 at least 100 colonies per plate clearly appeared. Without selecting colonies one by one, they were transferred to a fresh plate, and cultured in the presence of 0.01 μM MTX. A dozen or so colonies appeared. In this way, the concentration of MTX was increased to 0.02 μM, 0.05 μM and 0.1 μM to select surviving colonies. Alternatively, the colonies can also be selected by increasing MTX concentration after selecting the resultant a dozen or so colonies.

Moreover, a recombinant vector with the so-called polycistronic gene was prepared, and transfected in CHO cells. Thus, pAdD26SVpA was treated with PstI to recover two fragments, that were ligated with the CSF cDNA fragment derived from pBRV2, thereby constructing a recombinant vector with adenovirus promoter, CSF cDNA, DHFR and polyA site of SV40 (in this order). The vector was transfected into CHO cells.

Practical Example 22: Identification of activity of expressed G-CSF (-VSE)

From culture supernatants of the C127 and CHO cells obtained in Practical Examples 20 and 21, respectively, human G-CSF was obtained by the same method as described in Practical Example 16, and the activity of the human G-CSF was tested. The results are shown in Table 3.

Table 3: Testing of human G-CSF activity

		Human neutrophil colony number/dish
Purified human G-CSF (20 ng)		96
BPV	Culture supernatant of pdBPV-1-transfected C127 cells (concentrated 20-fold)	0
	Culture supernatant of pdBPV-1-transfected 3T3 cells (concentrated 20-fold)	0
	Culture supernatant of pTN-V2-transfected C127 cells (concentrated 20-fold)	107
	Culture supernatant of pTN-V2-transfected 3T3 cells (concentrated 20-fold)	103
dhfr	Culture supernatant of pAdD26SVpA-transfected CHO cells (concentrated 20-fold)	0
	Culture supernatant of pHGV2-dhfr-transfected CHO cells (concentrated 20-fold)	111
	Culture supernatant of pV2DR1-transfected CHO cells (concentrated 20-fold)	113

Practical Example 23: Amino acid analysis and carbohydrate analysis (-VSE)

1) Analysis of amino acid composition

The crude CSF sample obtained in Practical Example 22 was further purified by the method of (iii) of Practical Example 2. The purified CSF sample was subjected to amino acid composition analysis by the same method as described in 1) of Practical Example 17. The results are shown in Table 4.

Table 4 (Amino acid analysis table)

Amino acid	mol%
Asp (Asp+Asn)	2.3
Thr	4.0
Ser	8.1
Glu (Glu+Gln)	15.1
Pro	7.5
Gly	8.0
Ala	10.9
1/2 Cys	2.8
Val	3.9
Met	1.7
Ile	2.3
Leu	18.9
Tyr	1.7
Phe	3.5
Lys	2.3
His	2.9
Trp	1.2
Arg	2.9

2) Carbohydrate composition analysis

The above purified CSF sample used for amino acid analysis was also used for carbohydrate composition analysis by the same method and conditions as described in 2) of Practical Example 17. The analysis results confirmed the presence of galactose, N-acetylgalactosamine and sialic acid in the CSF of the present invention.

Practical Example 24: Construction of recombinant vector containing chromosome-derived gene for COS cells

The plasmid pBRCE3β containing the chromosomal gene shown in Fig. 5 obtained in Practical Example 11 was treated with EcoRI. On the other hand, the $pSVH^+K^+$ plasmid described by Bancrji et al. [Cell, Vol. 27, p. 299 (1981)] was treated with KpnI to remove the globulin gene. Furthermore it was partially digested with HindIII to remove a part of late phase genes of SV40. After re-ligation, the expression vector pML-E^+ was obtained.

This vector was treated with the restriction enzyme EcoRI and then de-phosphorylated with alkaline phosphatase (Takara Syuzo). The resultant vector DNA was ligated with the above chromosomal DNA fragment with T4 DNA ligase (Takara Syuzo), thereby obtaining pMLCE3α. As shown in Fig. 14, this plasmid is one

containing the enhancer of the SV40 gene, the replication initiation region of SV40, the replication initiation region of pBR322 and the β-lactamase gene derived from pBR322 (Amp^r). The chromosomal gene of human G-CSF was ligated downstream of the enhancer of SV40 gene.

Practical Example 25: Expression of chromosomal gene of human G-CSF in COS cells

10 mL of DMEM (Nissui Pharmaceuticals, Dalbecco-modified Eagle medium "Nissui") containing 10% fetal bovine serum was used. COS-1 cells (from Dr. Gluzman of Cold Spring Harbor Laboratory, USA) grown in a culture dish with a diameter of 9 cm (Nunc) to about 70% confluence were transfected using the phosphate-calcium method [Wigler et al.: Cell, Vol. 14, p. 725 (1978)] and DEAE-dextran : chlorochine method [e.g. Gordon et al.: Science, Vol. 228, p. 810 (1985)].

The phosphate-calcium method was performed as follows.

160 μg of the vector pMLCE3α prepared in Practical Example 24 was dissolved in 320 μL of TE solution. 3.2 mL of distilled water was added, and then 504 μL of 2 M $CaCl_2$ was added.

To this solution, 4 mL of 2 x HBS [50 mM Hepes, 280 mM NaCl, 1.5 mM phosphate buffer (pH 7.12)] was added. After cooling on ice for 20-30 min, 1 mL was added per culture dish with grown COS-1 cells. After culture at 37°C for 4 hr in CO_2 incubator, the cells were washed with serum-free DMEM medium. Subsequently, 5 mL of DMEM medium containing 20% glycerol was added. After allowing to stand at room temperature for about 3 min, the cells were washed again with serum-free DMEM medium. After removing the serum-free DMEM medium, 10 mL of DMEM medium containing 10% fetal bovine serum was added. After overnight culture in a CO_2 incubator, the medium was changed to a fresh one and the culture was further continued for 3 days.

The DEAE-dextran : chlorochine method was performed as follows.

As with the phosphate-calcium method, COS-1 cells were cultured to about 70% confluence, and then washed twice with serum-free DMEM medium. Serum-free DMEM medium containing DEAE-dextran at 250 μg/mL and the plasmid pMLCE3α

prepared in Practical Example 24 at 2 μg/mL were added. After culture at 37°C for 12 hr, the cells were washed twice with serum-free DMEM medium. Subsequently, culture was performed in DMEM medium containing 10% fetal bovine serum and 1 mM chlorochine at 37°C for 2 hr. The cells were washed twice again with serum-free DMEM medium. DMEM medium containing 10% fetal bovine serum was added, and the culture was further continued at 37°C for 3 days.

The culture supernatant of the COS-1 cells thus obtained was adjusted to pH 4 with 1 N acetic acid. An equal volume of n-propanol was added. The resultant precipitate was removed by centrifugation, and the sample was applied to an open column (1 ϕ x 2 cm) packed with a C8 reverse phase carrier (Yamamura Chemicals). Elution was performed with 50% n-propanol. The eluate was diluted 2-fold with water, and then applied to reverse phase high performance liquid chromatography using a YMC-C8 column (Yamamura Chemicals). Elution was performed with a linear concentration gradient of n-propanol at 30-60% in 0.1% TFA. Fractions at an n-propanol concentration of about 40% were collected and freeze-dried, followed by dissolution in 0.1 M glycine buffer (pH 9). By these procedures, human G-CSF was enriched about 20-fold from the supernatants of COS-1 cells.

For control, pML-E$^+$ containing no chromosomal gene of human G-CSF was transfected in COS-1 cells by the above method and the culture supernatant was concentrated.

The resultant samples were subjected to human G-CSF assay by the method described in the Reference Example "Assay for human G-CSF (a)". The results are shown in Table 5.

Table 5

	Human neutrophil colony number/dish
Purified human G-CSF (20 ng)	18
Culture supernatant of pML-E$^+$ transfected COS cells (concentrated 20-fold)	0
Culture supernatant of pMLCE3α-transfected COS cells (concentrated 20-fold)	23
Culture supernatant of pMLCE3α-transfected COS cells (10-fold concentrated)	19

Practical Example 26: Expression of chromosomal gene of human G-CSF in C127 cells

pMLCE3α plasmid obtained in Practical Example 24 was treated with EcoRI to recover an about 4 kb fragment by the method described in the above mentioned Molecular Cloning, as source of chromosome-derived G-CSF gene.

This was treated with Klenow fragment of DNA polymerase to prepare a blunt end (A).

On the other hand, from pHGA410 plasmid obtained in Practical Example 12, by the same method described in the above mentioned Molecular Cloning, the SV40 promoter region (an about 0.4 kb EcoRI-EcoRI fragment) was cut out and then treated with Klenow fragment of DNA polymerase (B).

Furthermore, the plasmid pdBPV-1 having bovine papilloma virus (BPV) [Sarver, M., Sbyrne, J. C. & Howley, P. M. (1982) Proc. Natl. Acad. Sci. USA, Vol. 79, p. 7147-7151; from Dr. Howley] was treated with HindIII and PvuII to obtain an about 8.4 kb DNA fragment. This was treated with the Klenow fragment of DNA polymerase and then dephosphorylated with bacterial alkaline phosphatase (C).

The above (A), (B) and (C) DNAs, each 0.1 μg, were dissolved in 20 μL of a reaction solution (50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP), and 180 units of T4 DNA ligase were added, followed by reaction overnight at 4°C.

This reaction solution was used to obtain pTNCE3α plasmid using the rubidium chloride method described in the above-mentioned Molecular Cloning.

As the source of chromosome-derived G-CSF gene, besides the above (A), a DNA fragment prepared as follows can also be used. Thus, 20 μg of pMLCE3α was dissolved in 100 μL of a reaction solution consisting of 10 mM Tris-HCl (pH 8.0), 7 mM $MgCl_2$, 100 mM NaCl, 7 mM 2-mercaptoethanol, 0.01% BSA), and 20 units of StuI were added, followed by incubation at 37°C for 5 hr. Using 1.2% agarose gel electrophoresis, an about 1.78 kb DNA fragment was obtained.

The pTNCE3α plasmid thus obtained was transfected in mouse C127 cells as in Practical Example 14 for expression, and clones with high G-CSF production were selected.

Practical Example 27: Expression of chromosomal gene of human G-CSF in CHO cells

As with C127 cells in Practical Example 26, the pMLCE3α plasmid was treated with StuI to recover an about 1.78 kb DNA fragment, or with EcoRI to recover an about 4 kb EcoRI fragment as source of chromosome-derived G-CSF gene.

This was treated with the Klenow fragment of DNA polymerase to prepare a blunt end (a).

On the other hand, as in Practical Example 26, from pHGA410 plasmid the SV40 promoter region (an EcoRI-EcoRI fragment) was cut out to obtain an about 0.4 kb fragment, that was similarly treated with the Klenow fragment of DNA polymerase (b).

Furthermore, the pAdD26SVpA plasmid [Kaufman, R. G. & Sharp, P. A. (1982) Mol. Cell. Biol. Vol. 2, p. 1304-1319] was treated with EcoRI, and then with Klenow fragment of DNA polymerase, followed by treatment with bacterial alkaline phosphatase for dephosphorylation (c).

The above (a), (b) and (c), each 0.1 μg, were dissolved in 20 μL of a reaction solution (50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP), and 180 units of T4 DNA ligase was added, followed by reaction overnight at 4°C.

This reaction solution was transformed in E. coli strain DH1 by the rubidium chloride method described in the above mentioned Molecular Cloning. Tet^r colonies were obtained, thereby obtaining the plasmid pAdD26SVpACE3α.

The plasmid pAdD26SVpACE3α had the CSF gene ligated with the SV40 initial gene and had DHFR gene downstream of the adenovirus major late phase promoter.

On the other hand, pAdD26SVpA was treated with EcoRI and BamHI as described in 2) of Practical Example 15. A resultant about 2 kb DNA fragment

containing DHFR gene was ligated with the above (a) and the EcoRI-SalI fragment of pHGA410 (H) to construct the Amp^r expression vector pDRCE3α (Fig. 15).

The pAdD26SVpACE3α and pDRCE3α plasmids were transfected in CHO cells as in Practical Example 15. MTX selection was repeated to obtain G-CSF-producing strains.

Practical Example 28: Identification of the activity of expressed G-CSF (human chromosome-derived gene)

From culture supernatants of the C127 and CHO cells obtained in Practical Examples 26 and 27, respectively, human G-CSF was obtained by the same method as described in Practical Example 25, and the activity of the human G-CSF was tested. The results are shown in Table 6.

Table 6: Testing of human G-CSF activity

		Human neutrophil colony number/dish
Purified human G-CSF (20 ng)		85
BPV	Culture supernatant of pdBPV-1-transfected C127 cells (concentrated 20-fold)	0
	Culture supernatant of pTNCE3α-transfected C127 cells (concentrated 20-fold)	83
dhfr	Culture supernatant of pAdD26SVpA-transfected CHO cells (concentrated 20-fold)	0
	Culture supernatant of pD26SVCE3α - transfected CHO cells (concentrated 20-fold)	85
	Culture supernatant of pDRCE3α-transfected CHO cells (concentrated 20-fold)	86

Practical Example 29: Protective effect on human G-CSF on infection

<Test method>

1. Protective effect on Pseudomonas aeruginosa infection

Endoxan (Shionogi, trade name) was injected intraperitoneally at 200 mg/kg in 8-9 week old (body weight: 35.3 ± 1.38 g) ICR mice (male). The mice were divided into three groups. In two groups human G-CSF (25000 U/mouse or 5000 U/mouse) in a solution (1% propanol, 0.5% (w/v) mouse serum albumin) was injected subcutaneously, while in the third group the solution alone was injected. Injection was performed once every 24 hr for four times, each at 0.1 mL. Three hours after the fourth injection, Pseudomonas aeruginosa GNB-139 (3.9×10^5 CFU/mouse) was injected subcutaneously

for infection in all groups. At 21 hr after infection, human G-CSF (25000 U/mouse or 5000 U/mouse)-containing solution or solution alone was injected subcutaneously in the respective groups.

The protective effect on infection was investigated based on number of survived mice on day 10 after infection.

(Preparation of bacterial solution)

Hart Infusion Agar Plate (Difco, trade name) was used to culture Pseudomonas aeruginosa GNB-139 at 37°C overnight with shaking. The culture was suspended in physiological saline.

(The results)

(1) The purified human G-CSF from CHO cells (+VSE), the same sample as used for the amino acid composition analysis in Practical Example 17, was used for this testing.

The results are shown in Table 7.

Table 7: Effect on Pseudomonas aeruginosa

Group	CSF concentration (U/mouse/day)	Survived number/total number
Solution	0	0/10
CSF-containing solution	25000	9/10
CSF-containing solution	50000	10/10

Similarly, the purified human G-CSF from C127 cells, the same sample as used for the amino acid composition analysis in Practical Example 17, was used for testing the protective effect on infection, and roughly identical results were obtained.

(2) The purified human G-CSF from CHO cells (-VSE), the same sample as used for the amino acid composition analysis in Practical Example 23, was used for this testing.

The results are shown in Table 8.

Table 8: Effect on Pseudomonas aeruginosa

Group	CSF concentration (u/mouse/day)	Survived number/total number
Solution	0	0/10
CSF-containing solution	25000	9/10
CSF-containing solution	50000	10/10

Similarly, the purified human G-CSF from C127 cells, the same sample as used for the amino acid composition analysis in Practical Example 23, was used for testing the protective effect on infection, and roughly identical results were obtained.

[Effect of the Invention]

The glycoprotein of the present invention having the activity of human G-CSF is extremely useful as a therapeutic agent for the treatment of leukocytopenia, bone marrow leukemia, or for the enhancement of hematopoiesis, or for the prevention of infectious diseases.

Moreover, the establishment of the method for producing G-CSF desired as a therapeutic agent using gene recombination can be said to be a major accomplishment of the present invention.

4. Brief Legends to the Figures

Fig. 1 shows the sequences of probe (IWQ), probe (A) and probe (LC).

Fig. 2 shows the nucleotide sequence of the pHCS-1 insert.

Fig. 3 (A) shows the nucleotide sequence of pBRG4 cDNA insert.

Fig. 3 (B) (I) shows the amino acid sequence of the precursor of human G-CSF deduced from pBRG4 cDNA.

Fig. 3 (B) (II) shows the amino acid sequence of mature human G-CSF deduced from the pBRG4 cDNA.

Fig. 4 (A) shows the nucleotide sequence of pBRV2 cDNA insert.

Fig. 4 (B) (I) shows the amino acid sequence of the precursor of human G-CSF deduced from pBRV2 cDNA.

Fig. 4 (B) (II) shows the amino acid sequence of mature human G-CSF deduced from pBRV2 cDNA.

Fig. 5 shows the nucleotide sequence of human chromosomal gene encoding human G-CSF.

Fig. 6 shows the restriction enzyme sites of human G-CSF cDNA derived from pBRG4 or pBRV2.

Fig. 7 shows the restriction enzyme sites of human chromosomal gene encoding human G-CSF.

Fig. 8 shows a rough structure of pHGA410.

Fig. 9 shows the construction process for the recombinant expression vectors pTN-G4, pTN-G4VAα and pTN-G4VAβ.

Figs. 10a and 10b show the construction process for pHGG4-dhfr.

Fig. 10c shows the construction process for pG4DR1 and pG4DR2.

Fig. 11 shows a rough structure of pHGV2.

Fig. 12 shows the construction process for the recombinant expression vectors pTN-V2, pTN-VAα and pTN-VAβ.

Figs. 13a and 13b show the construction process for pHGV2-dhfr.

Fig. 13c shows the construction process for pV2DR1 and pV2DR2.

Fig. 14 shows a rough structure of pMLCE3α.

Fig. 15 shows rough structures of pD26SVCE3α and pDRCE3α.

Patent Applicant: Chugai Pharmaceuticals Corp., Ltd.
Agent: Patent Attorney Nozaki Tetsuya

Fig. 1

Probe (IWQ)

```
      Ile Trp Gln Gln Met Glu Glu Leu Gly Met
5'---- ATI TGG CAA CAA ATG GAA GAA CTI GGI ATG ----3'
           C   C       C   C T
```

Probe (A)

```
         Met Pro Ala Phe Ala
5'---- ATG CCA GCA TTT GC  ----3'
           T   T   C
           G   G
           C   C

3'---- TAC GGA CGA AAA CG  ----5'
           T   T   G
           G   G
           C   C
```

Probe (A)

Probe (LC)

Gln Glu Lys Leu Cys Ala Thr Tyr

5'---- CAG GAG AAG CTG TGT GCC ACC TAC ----3'

3'---- GTC CTC TTC GAC ACA CGG TGG ATG ----5'

Probe (LC)

Fig. 2

```
 CC  CTG  GAA  GGG  ATC  TCC  CCC  GAG
TTG  GGT  CCC  ACC  TTG  GAC  ACA  CTG
CAG  CTG  GAC  GTC  GCC  GAC  TTT  GCC
ACC  ACC  ATC  TGG  CAG  CAG  ATG  GAA
GAA  CTG  GGA  ATG  GCC  CCT  GCC  CTG
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CGG  GCA  GGA  GGG  GTC  CTA  GTT  GCC
TCC  CAT  CTG  CAG  AGC  TTC  CTG  GAG
GTG  TCG  TAC  CGC  GTT  CTA  CGC  CAC
CTT  GCC  CAG  CCC  TGA  GCC  AAG  CCC
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```
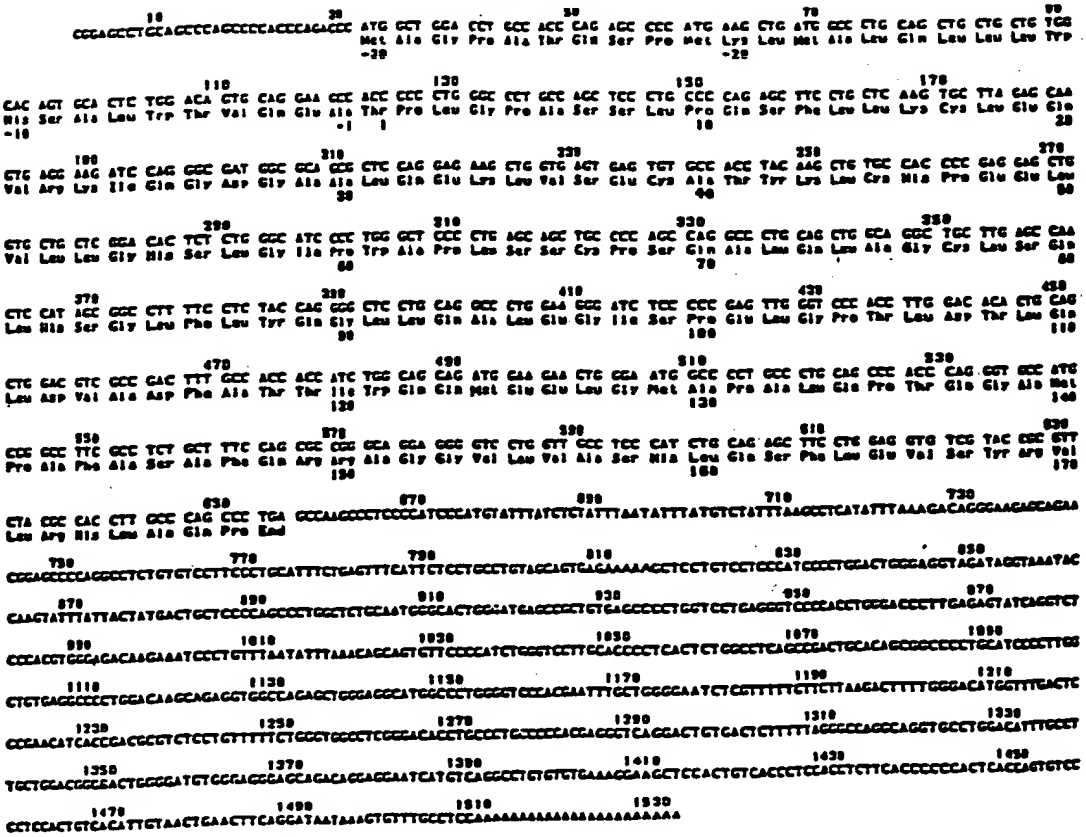

Fig. 3 (A)



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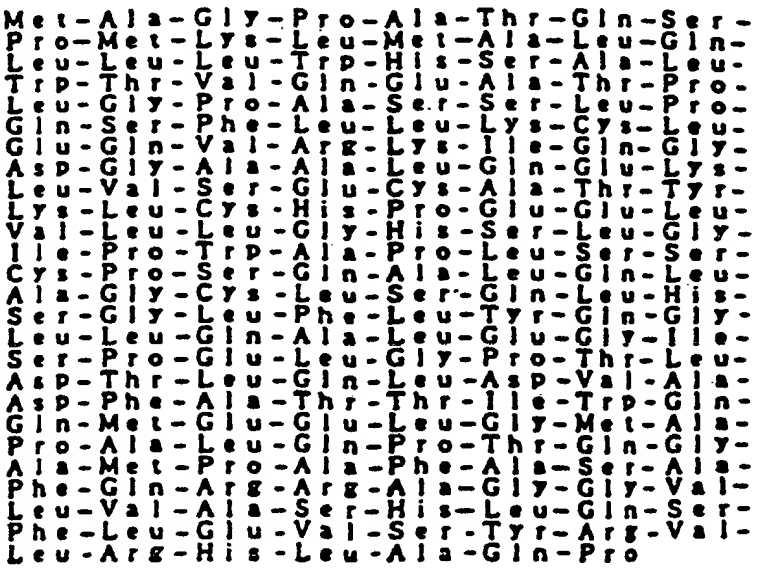
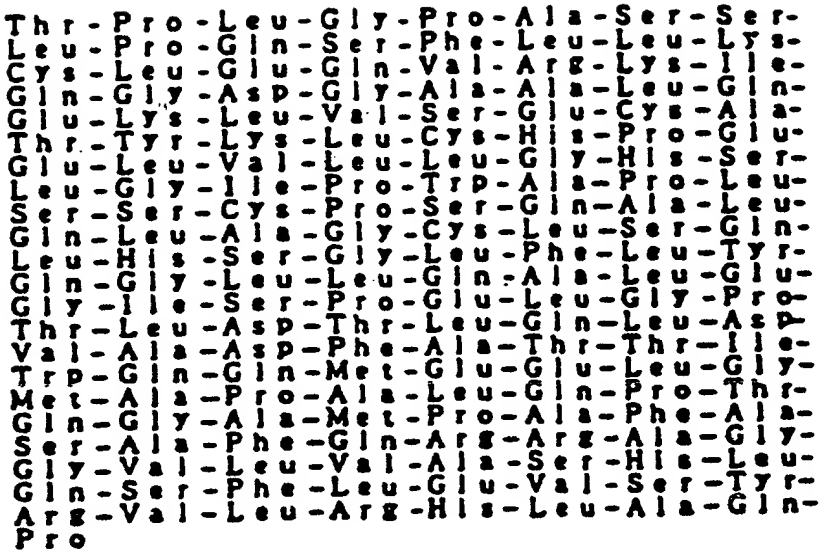


Fig. 3 (B)



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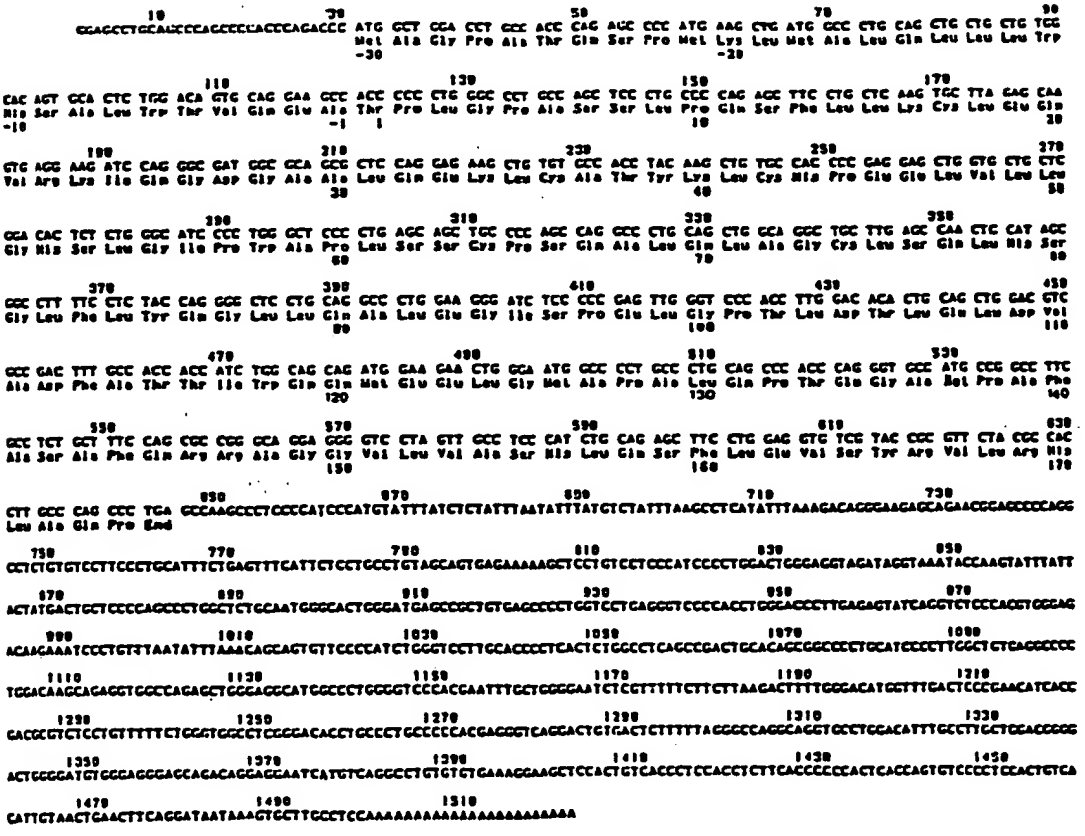
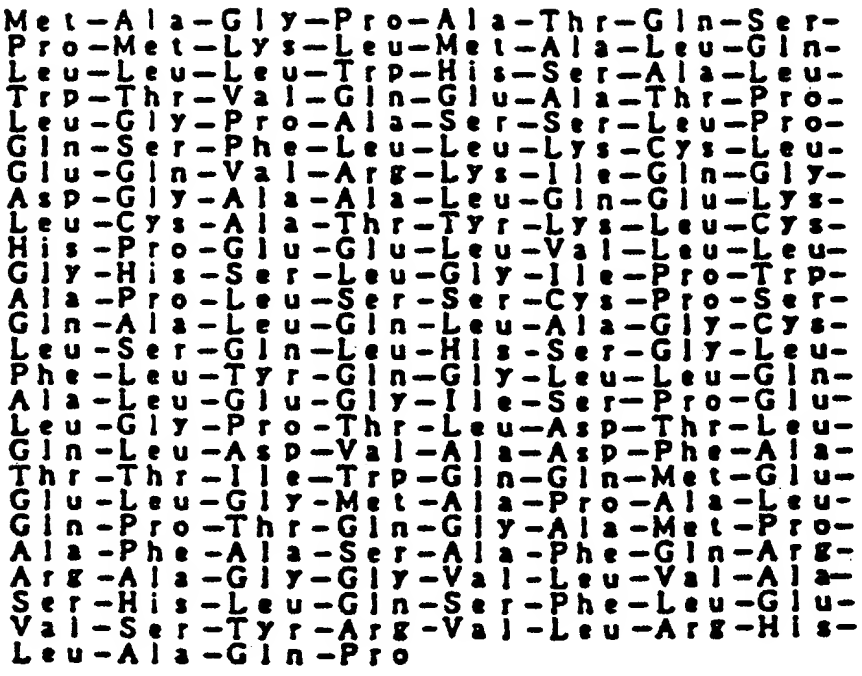
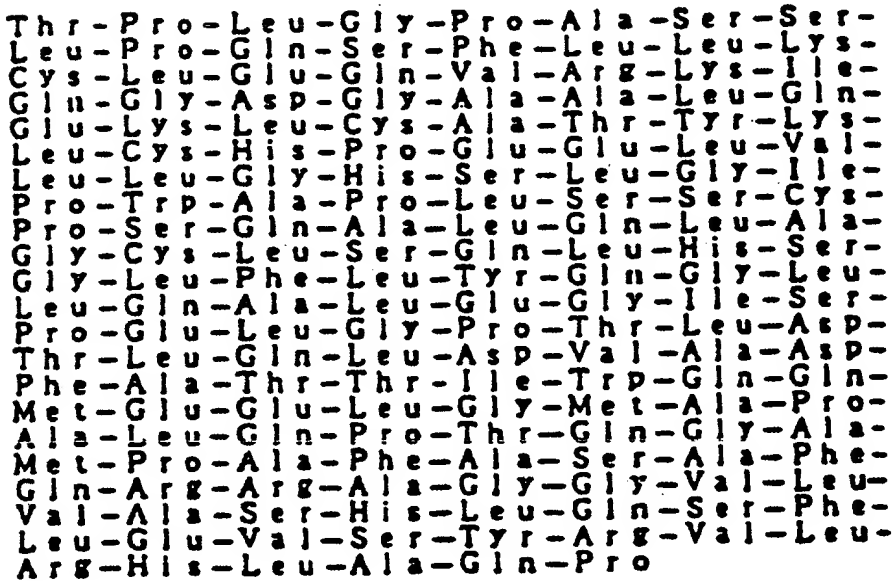


Fig. 4 (B)



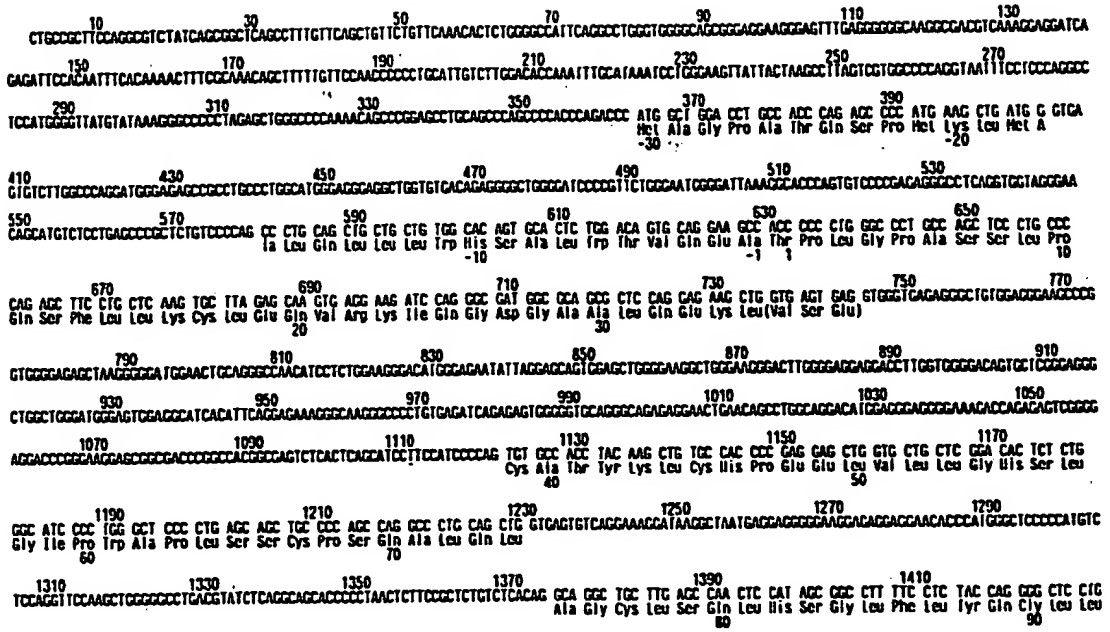
(I)

Fig. 4 (B)



(II)

(Attachment-1) Fig. 5-1



(Attachment-2) Fig. 5-2

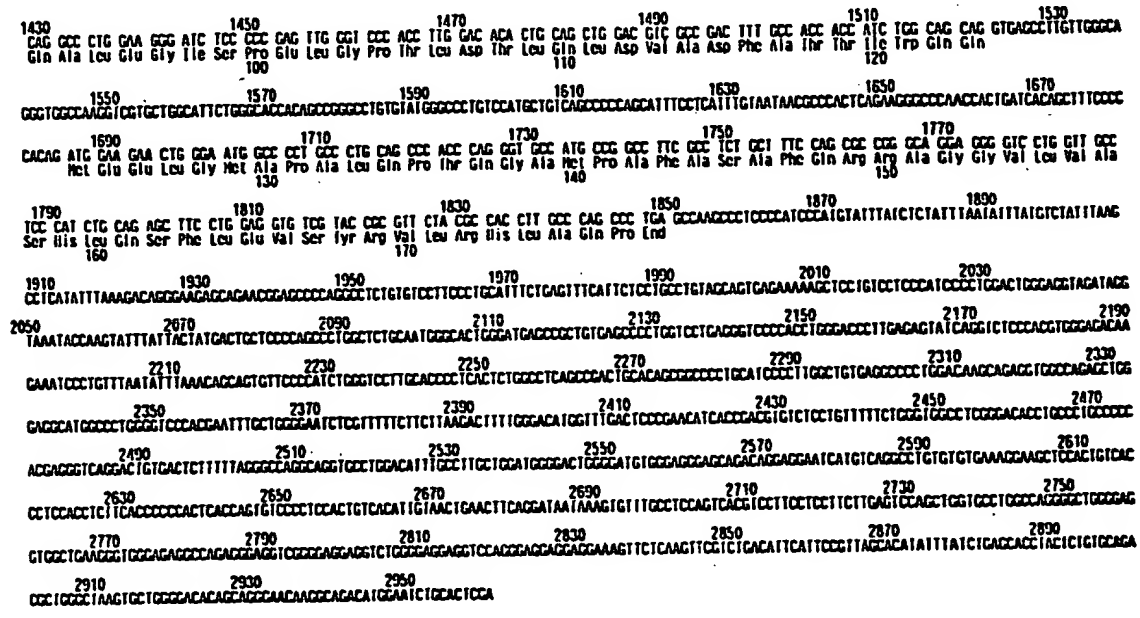


Fig. 6

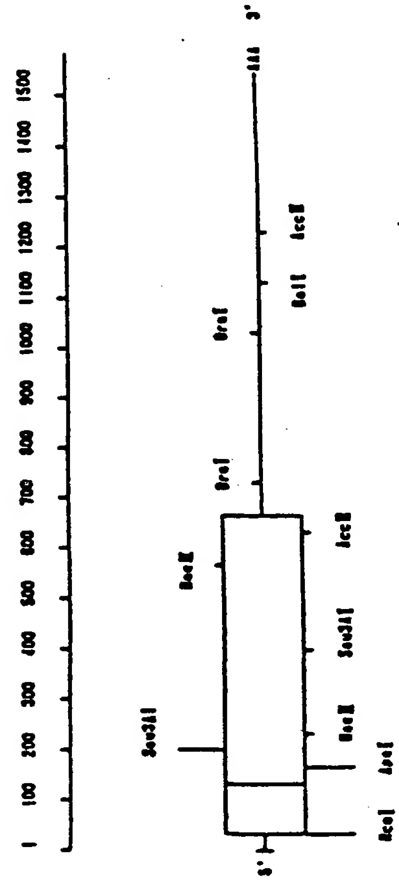


Fig. 7

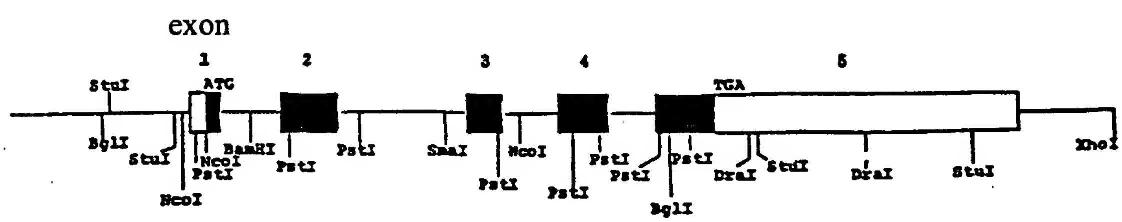

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Fig. 8

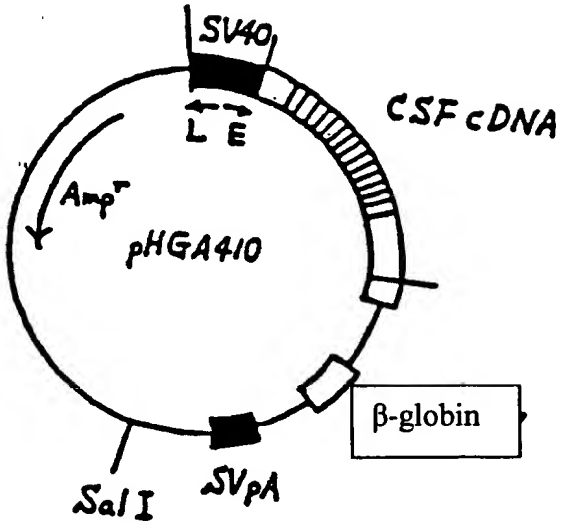

Fig. 9

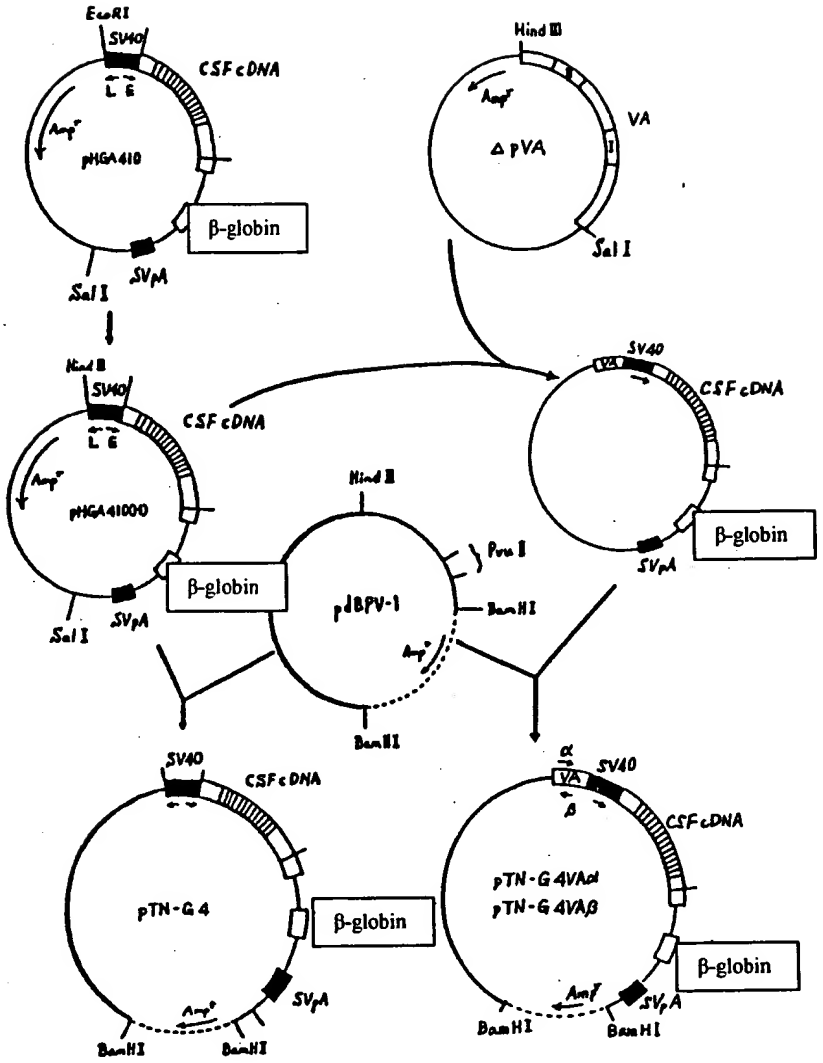
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Fig. 10a

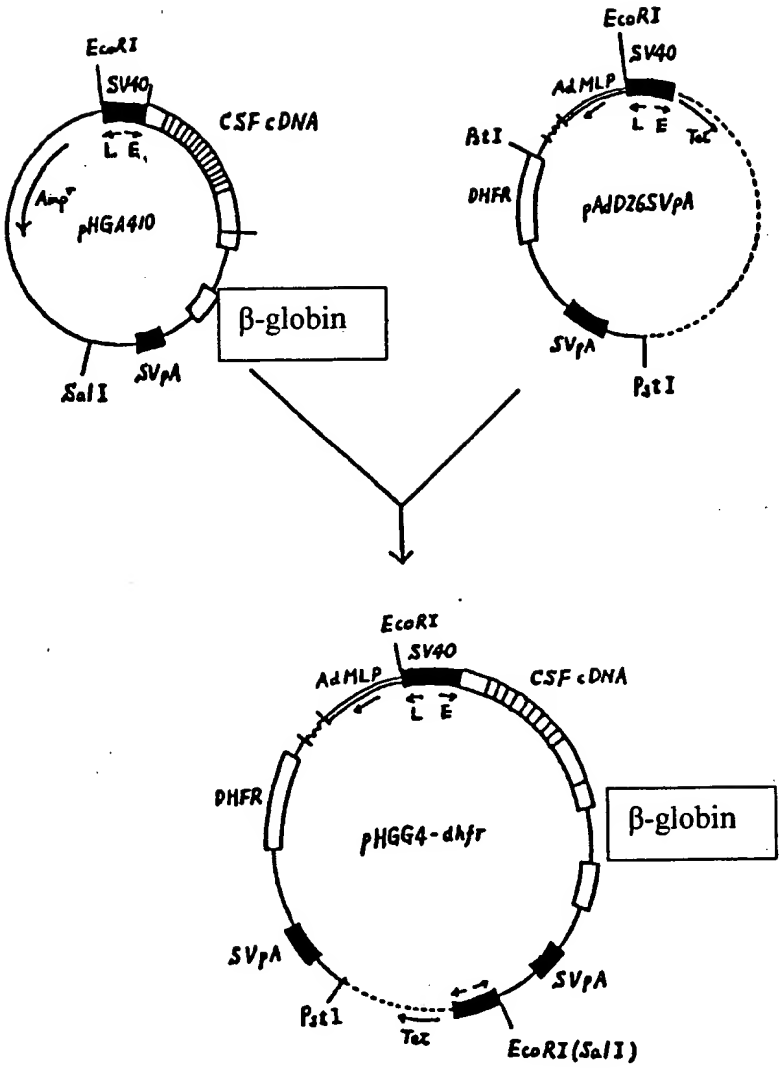
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Fig. 10b

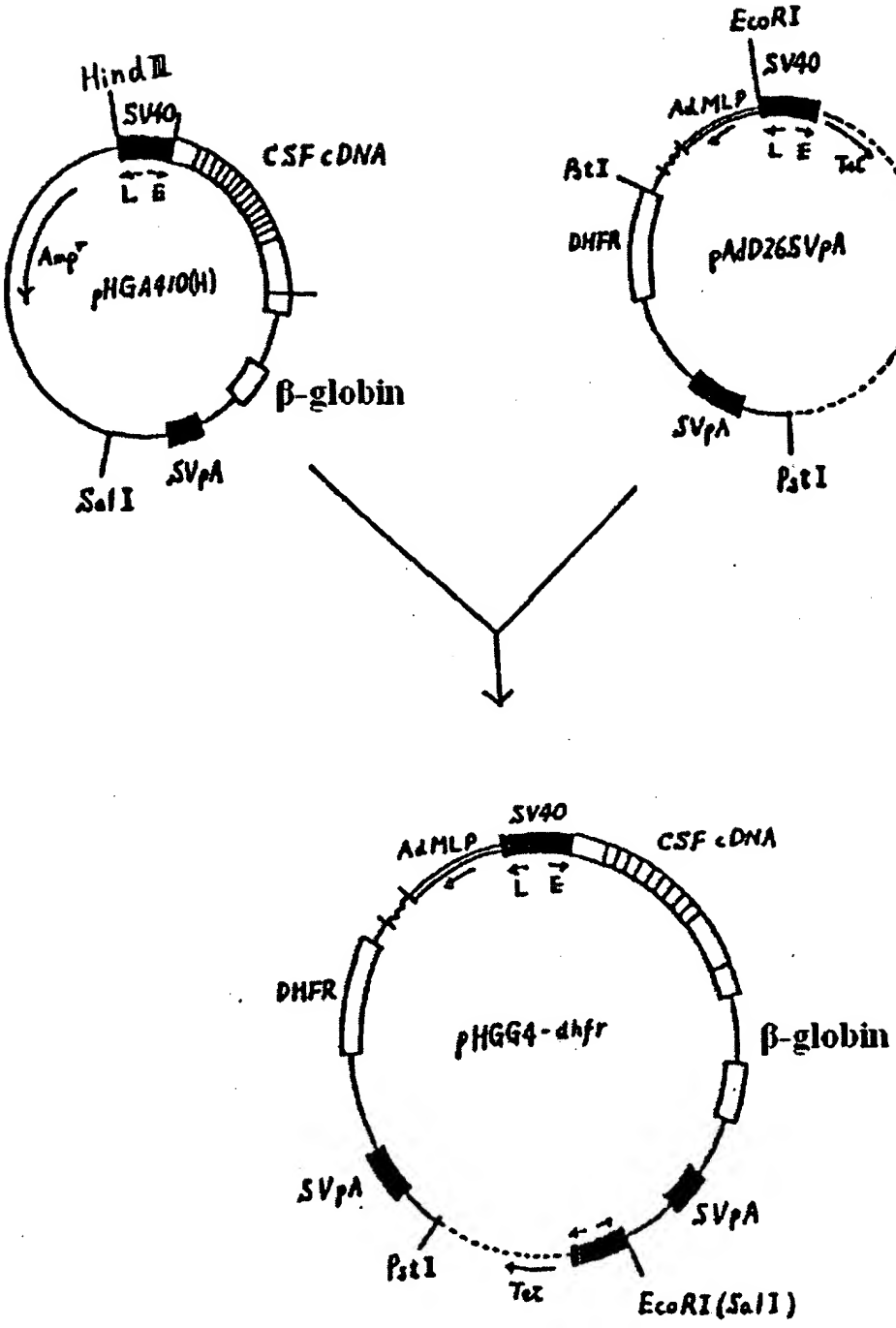
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Fig. 10c

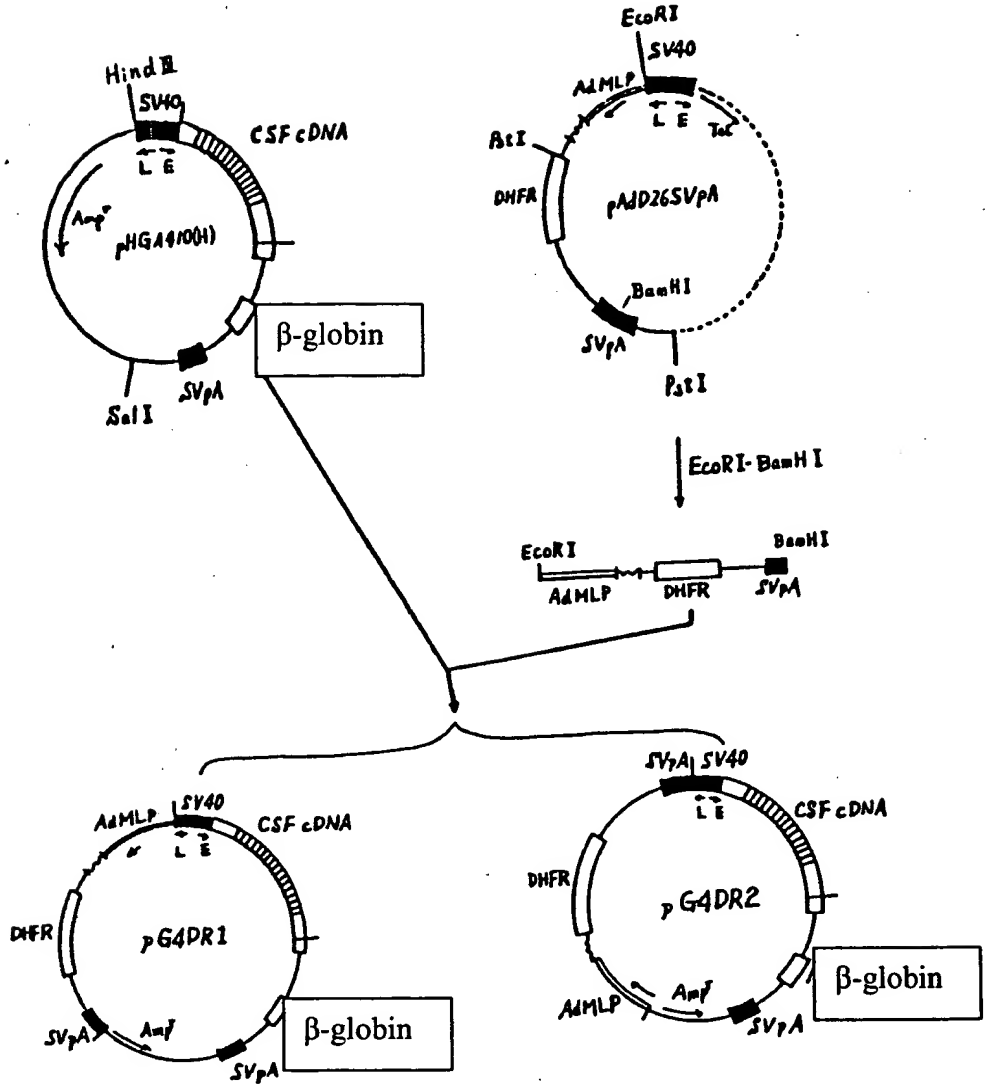
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Figure 11

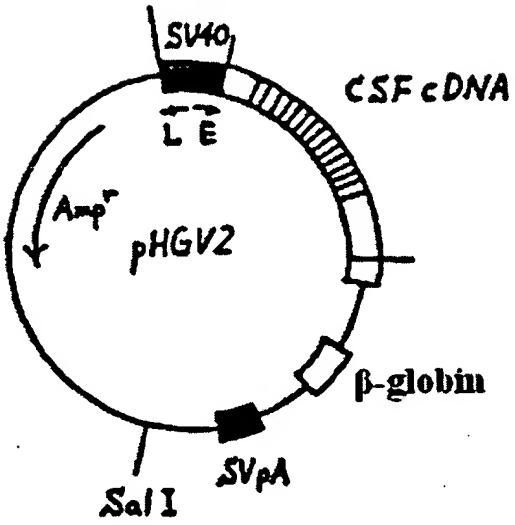

Figure 12

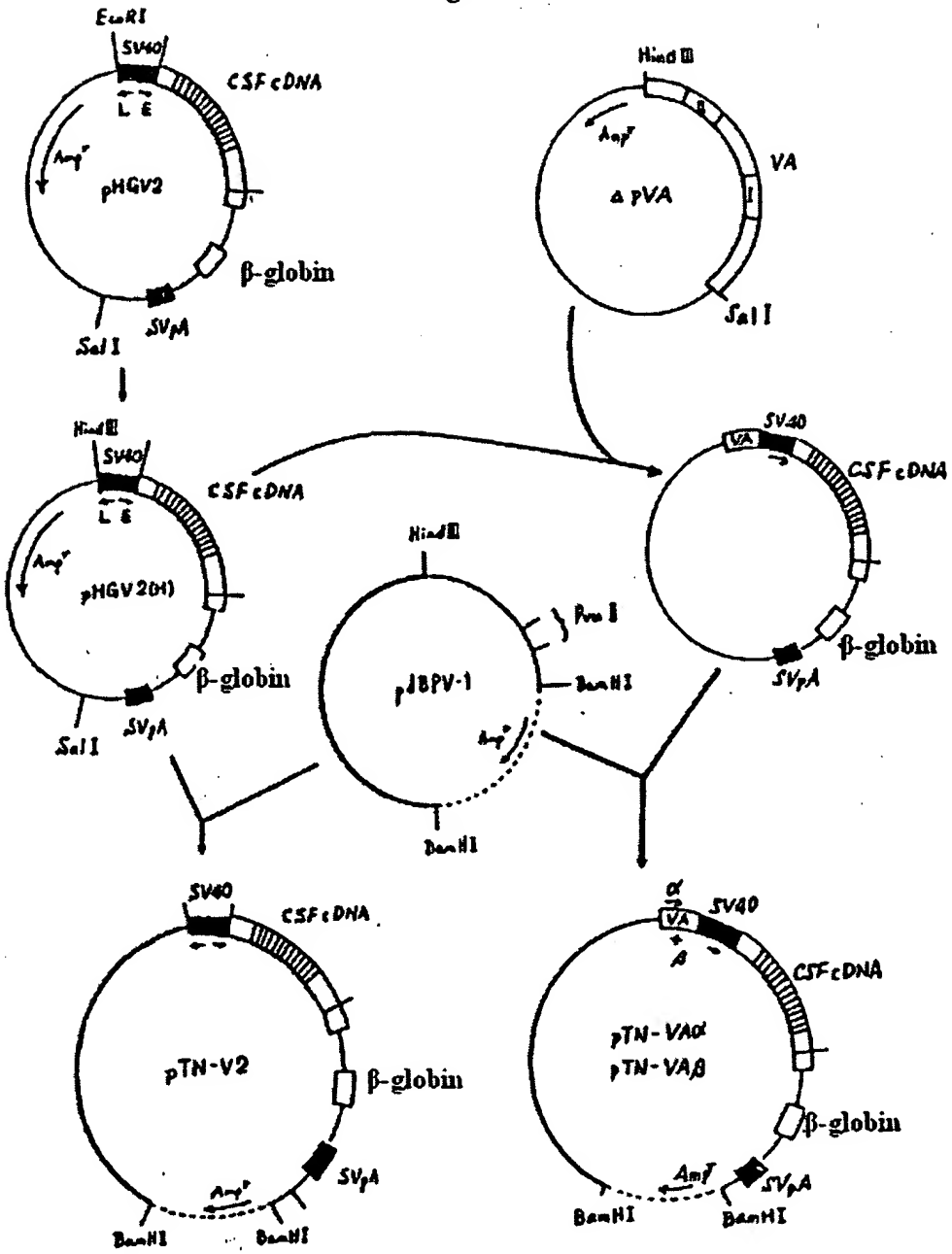
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Figure 13a

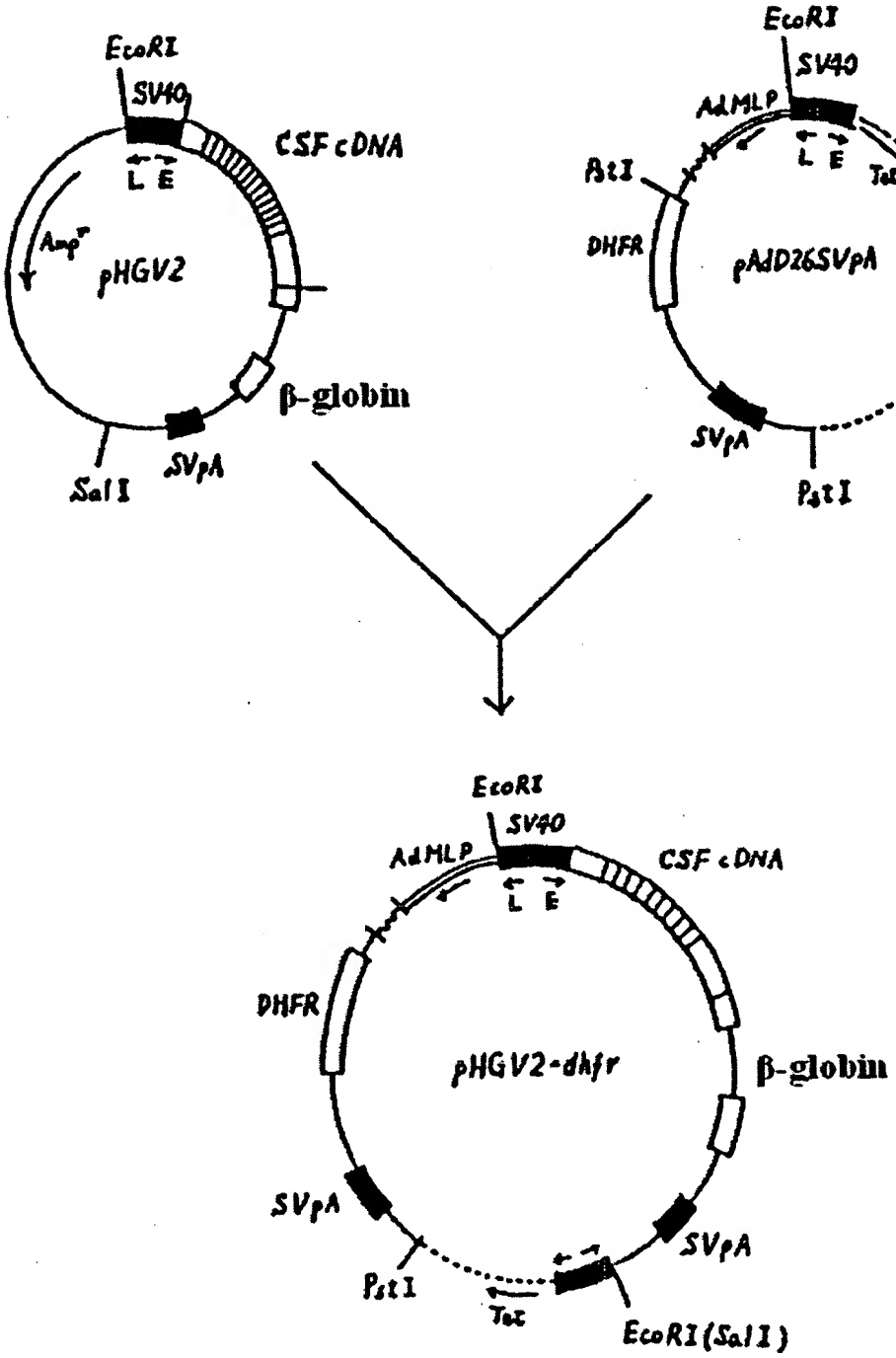
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Figure 13b

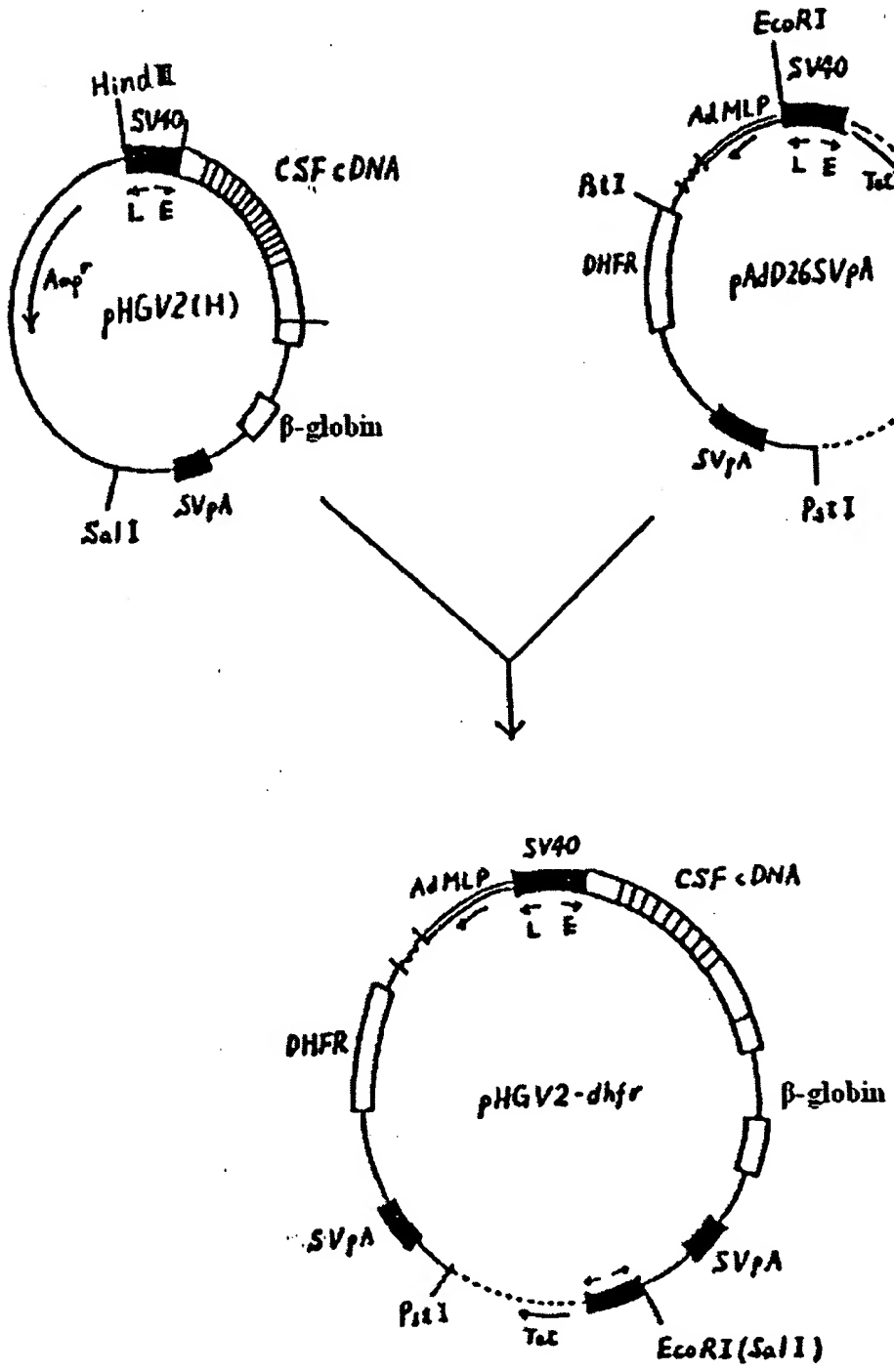
HindIII
SV40
CSF cDNA
L E
Amp^r
pHGV2(H)
β-globin
SVpA
SalI
EcoRI
SV40
AdMLP
L E
Tat
BtI
DHFR
pAdD26SVpA
SVpA
PstI
SV40
AdMLP
CSF cDNA
L E
DHFR
pHGV2-dhfr
β-globin
SVpA
SVpA
PstI
Tat
EcoRI(SalI)

Figure 13c

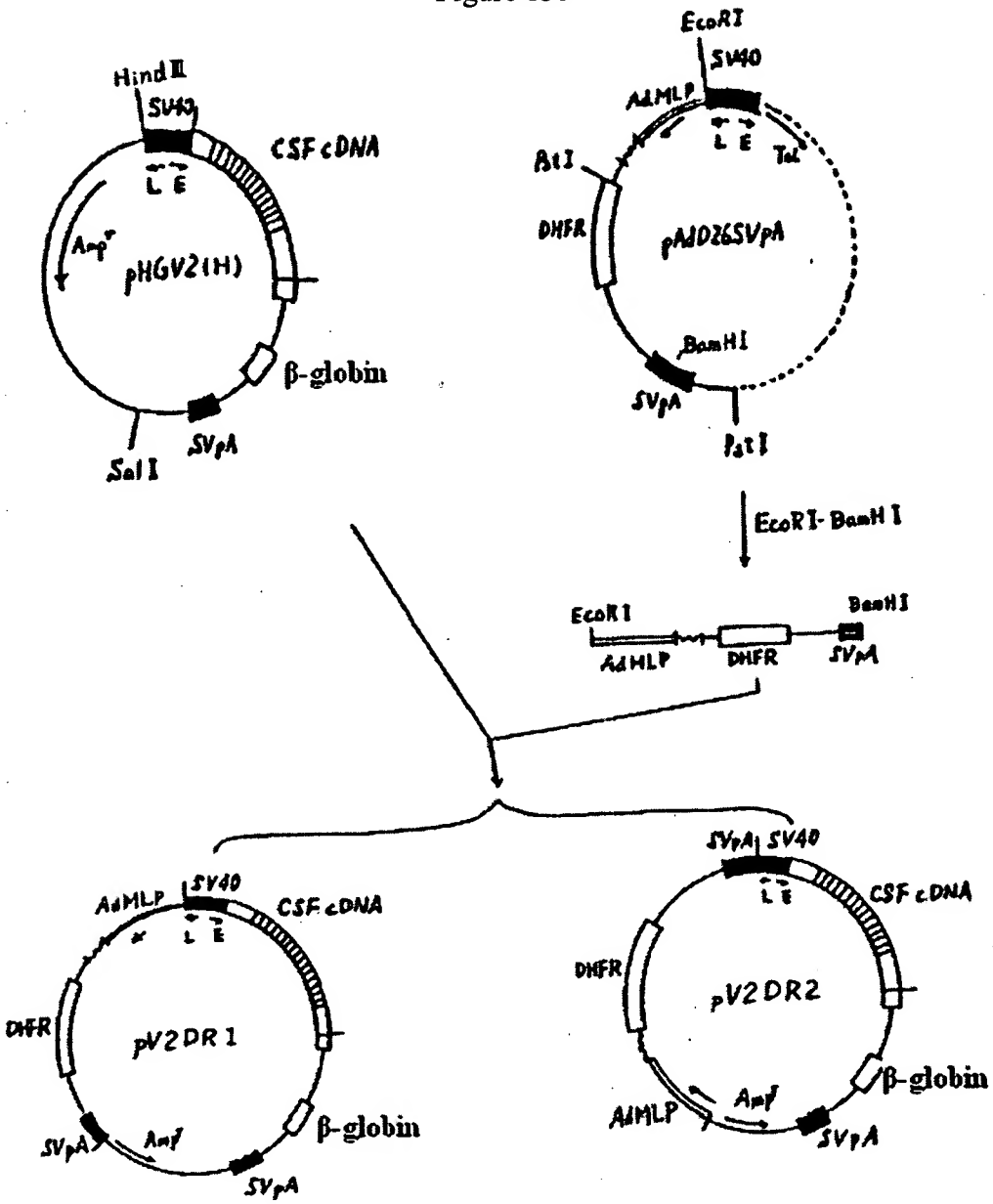
Hind III
SV40
CSF cDNA
L E
Amp
pHGV2(H)
β-globin
SVpA
Sal I
EcoRI
SV40
AdMLP
L E
Tpl
BstI
DHFR
pAD26SVpA
BamHI
SVpA
Pst I
EcoRI- BamH I
EcoR I
BamHI
AdMLP
DHFR
SVpA
SVpA
SV40
CSF cDNA
AdMLP
SV40
L E
DHFR
pV2DR1
SVpA
Amp
β-globin
SVpA
SVpA
SV40
L E
CSF cDNA
DHFR
pV2DR2
β-globin
AdMLP
Amp
SVpA

Figure 14

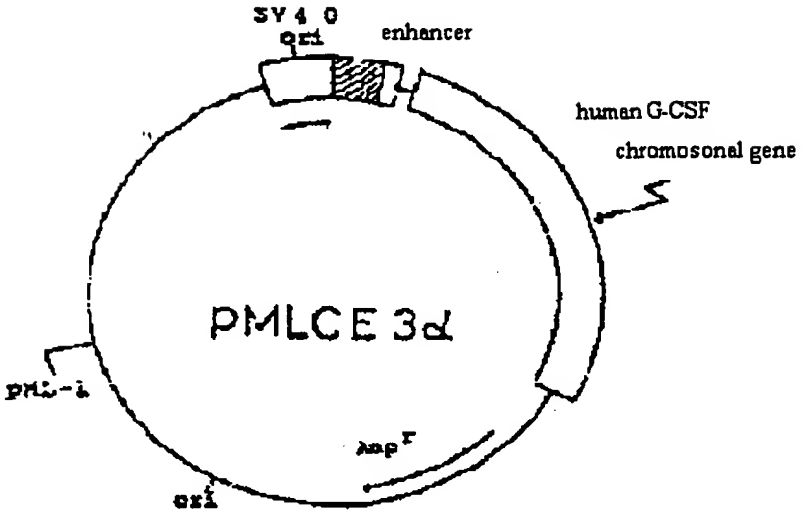

Figure 15